Biochemical Studies on Heparin-like Glycosaminoglycans from Basophils and Mast Cells in Allergy and Anaphylaxis.

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A Thesis presented for the degree

of

Doctor of Philosophy

of the

University of Edinburgh

in the

Faculty of Medicine

1987

Abstract.

The involvement of basophils in IgE mediated allergic disease is well recognized, but their exact role is still unclear. The release of histamine from the secretory granules has been extensively used in the study of basophils in allergy: since histamine is complexed with glycosaminoglycans (GAGs) in the secretory granules, the release of granule-derived GAG from basophils was studied in relation to that of histamine to examine further the mechanisms and involvement of basophils in anaphylactic type hypersensitivity.

A competitive binding assay was developed for the detection of naturally occurring GAGs, which were found to be contained only in the basophil fraction of whole blood. GAG release from basophil enriched fractions of whole blood of both atopic and non-atopic individuals was Ca^{++} and dose dependent after challenge with Dermatophagoides pteronyssinus, and differed markedly from histamine release both in time course and allergen concentration required for optimum release. The major highly sulphated GAGs contained in human basophils and rat mucosal mast cells were identified as heparan sulphate and chondroitin sulphate E; these were released, together with histamine and rat mast cell protease II during systemic anaphylaxis of Nippostrongylus brasiliensis -primed rats challenged intravenously with worm antigen.

Basophil counts in atopic individuals were significantly elevated above those of non-atopic individuals, as were eosinophil counts, which paralleled those of basophils in both populations. Basophil counts did not fluctuate over short or long time intervals, and did not correlate with plasma GAG levels in either population. Individual plasma GAG levels fluctuated considerably, and did not

differ significantly between the two populations. However, in a group of patients with chronic myelogenous leukaemia, with very high basophil counts, plasma GAG levels were raised and there was significant correlation of plasma GAG and circulating basophil numbers.

GAG content of mast cells in nasal turbinates of atopic patients was less than that of non-atopic controls, indicating mast cell GAG involvement associated with granule release in atopic disease.

Thus, the release of highly sulphated GAG from normal human basophils has been characterized, and used to study their involvement in type 1-anaphylactic hypersensitivity reactions. It should prove valuable in further investigation of the physiology and pathophysiology of basophils.

Acknowledgements.

I would like to thank my three supervisors for their guidance and encouragement throughout the course of this work; Dr R StC Barnetson (Department of Dermatology, University of Edinburgh), Dr PL Yap (South East Scotland Blood Transfusion Service), and especially Dr J Dawes for the use of the facilities in her department at the MRC/SNBTS Blood Components Assay Group, Edinburgh.

Much of the work described in this thesis was performed in collaboration with other groups, and I would like to thank all those involved, namely:

Dr HRP Miller and Dr SJ King (Moredun Research Institute, Edinburgh), who provided the opportunity for the study of anaphylaxis in the animal model.

Dr EC Benton for collecting samples from atopic patients at her out-patient clinic at Department of Dermatology, Royal Infirmary, Edinburgh, and Mr HAF MacFarlane for arranging sample collection, providing all IgE measurements and clinical details of these patients, and for his assistance with statistical analyses.

Dr TL Allen and Mr F MacKenzie, Department of Haematology, Law Hospital, Lanarkshire, who performed all basophil counts.

Dr JA Wilson (Department of Otolaryngology, University of Edinburgh), who provided nasal turbinate samples for the study of nasal tissue mast cells, and Dr DM Salter (Department of Pathology, University of Edinburgh) who performed histology and cell counts on these tissue samples.

Dr J Craig, who provided samples from patients with leukaemic disorders, and from patients receiving platelet transfusions.

Dr DM Robertson and Dr CS Hosking (Department of Immunology, Royal Childrens Hospital, Victoria, Australia), for providing samples to study adverse reactions in patients receiving intravenous immunoglobulin infusions.

I would also like to thank Dr A Westwood (Department of Paediatric Biochemistry, Royal Hospital for Sick Children, Edinburgh) for allowing me to use the facilities in his department for histamine assays, and to Mr T Healy for his technical advice; Dr EB Mitchell and his staff (Department of Clinical Immunology, Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex) for inviting me to visit his laboratory to learn basophil purification techniques, and valuable comments concerning many aspects of this work; Dr IR MacGregor (Scottish National Blood Transfusion Service Headquarters Laboratory, Edinburgh), who performed density gradient centrifugation; Miss S Maguire (MRC/SNBTS Blood Components Assay Group) for her assistance with gel filtration; and to the Scottish Home and Health Department who provided the financial support for this project.

My final thanks are to my parents, for all their help and support throughout the course of my education.

Declaration

I hereby declare that this thesis does not include work submitted for any other degree or professional qualification of this or any other University or Institution of learning.

This thesis has been composed by myself and the work described in it is my own.

Karen M Reilly

Dedication

I dedicate this thesis to my parents, Gerald and Mary Reilly.

Contents

Chapter 1.

General Introduction.

Allergy is a complicated subject, and an immunological enigma. The term was originally coined by von Pirquet in 1906 to describe an altered immune reactivity of an animal, following exposure to a foreign antigen, and included both immunity and hypersensitivity. In Britain, allergy and hypersensitivity are used synonymously, whereas in some parts of the world such as Scandanavia, allergy refers only to IgE mediated hypersensitivity. Four main types of hypersensitivity reactions are recognized, as classified by Coombs and Gell in 1963: Type 1 - anaphylactic hypersensitivity, type 2 - cytotoxic (cytolytic) hypersensitivity, type 3 - immune complex mediated hypersensitivity, and type 4 - delayed type hypersensitivity. The subject of this thesis is related to anaphylactic type hypersensitivity.

The role of type 1 hypersensitivity from a teleological point of view is hard to define. It seems likely that IgE forms part of the immune protection against helminths; it must be remembered that more than half the world population suffers from helminthic disease, and such people have high concentrations of IgE in their blood (Ezeoke et al 1973). Helminthic infestation is not a problem in the Western world, but despite this 15-20% of the population of Western countries produce significant amounts of IgE to inhalant and food allergens, and such people are termed "atopic" (Coca and Cooke 1923); clinically they may suffer from allergic asthma, allergic rhinitis, allergic conjunctivitis, IgE mediated food allergy, and atopic eczema. Though atopic individuals are prone to

developing generalized anaphylactic reactions in certain circumstances, it is probable that everyone can develop anaphylaxis to antigens such as drugs or Hymenoptera venoms.

Anaphylaxis is one of the most important medical emergencies, and inadequate therapy may result in the death of the patient from profound hypotension, glottal oedema or severe bronchospasm. The term anaphylaxis was introduced by Portier and Richet (1902) who noted that repeated injections of foreign protein might induce not protection, but injury and even death. They injected Actinaria (marine creatures) into dogs and noted that the animals, instead of becoming increasingly immune to repeated doses, developed progressive illness, that is, loss of "phylaxis" or anaphylaxis. Subsequently, Prausnitz (Prausnitz and Kustner 1921) showed that a humoral factor in allergic serum could be passively transferred to a second non-allergic individual, with resultant reaction to that allergen (in this case to fish protein). It was not until the 1960s that the exact nature of the underlying immunological events of type 1 hypersensitivity reactions were further elucidated by the work of the Ishizakas and that of Johannson and Bennich. The Ishizakas demonstrated from studies on ragweed allergic rhinitis that there was a heat-labile immunoglobulin present in patients' serum which was not IgG, IgA, IgM or IgD, and they named it IgE (Ishizaka and Ishizaka 1966; Ishizaka et al 1966; Ishizaka and Ishizaka 1967). Their findings were complemented by those of Johannson and Bennich who recognized that one of their patient's myeloma was not IgG, IgA, IgM or IgD (Johansson and Bennich 1967) and named the immunoglobulin IgND: they also recognized that patients with asthma had raised levels of this immunoglobulin in their serum (Johansson 1967). Collaborative studies between the

Ishizakas and the Scandanavian work confirmed finally that IgE and IgND were one and the same antibody (Bennich et al 1969).

IgE is a monomeric divalent immunoglobulin with two light and two heavy (ε) polypeptide chains and does not fix complement via the classical pathway, but may activate C_3 and later components of the complement sequence by means of the alternative pathway (Ishizaka and Ishizaka 1973). Like all other immunoglobulins, IgE is produced by plasma cells, and has a molecular weight of 188,000, 12% of which is carbohydrate. In the Western world it is a minor component of circulating immunoglobulins, present in concentrations of 0-400ng/ml, although atopic individuals may have up to 1000 times this amount. It has a half life in human serum of 2-3 days (Waldmann 1969; Waldmann et al 1976), which is relatively short compared with other immunoglobulins, such as IgG (which has a half life of 25 days).

A feature of fundamental importance to IgE function is its ability to bind avidly and exclusively to the surface of two cell types: basophils and mast cells. Binding is reversible and does not involve a covalent bond (Ishizaka et al 1973; Ishizaka and Ishizaka 1974). These cells contain specific membrane receptors with high binding affinities for the F_c portion (Ishizaka et al 1973; Ishizaka et al 1979) of IgE molecules (10^{-8} to 10^{-9} M for human basophils). A normal human basophil is estimated to have 30-100 x 10^3 receptor sites, most of which are filled in an atopic individual, and is thought to bind 10-40 x 10^3 IgE molecules, which may be increased by 3-7 times, with exposure to high concentrations of IgE. Cross-linking of adjacent IgE receptors on mast cell and basophil surfaces by whatever means, results in the release of various mediators stored within the cytoplasmic granules, which is

a highly complex sequence of events (simplified in Figure 1), and produces the symptoms typical of immediate hypersensitivity or allergic reactions.

The elevated serum levels of IgE which initiate allergic reactions in sensitised individuals also occur in response to various intestinal parasitic infections (Ezeoke et al 1973). In all its forms, allergy is regarded as a disease state, but the mast cell/basophil/IgE antibody system can be beneficial as in establishment of immunity to certain parasitic infections, particularly intestinal helminths. IgE antibodies produced in response to exposure to helminth antigen activate the mast cell/basophil system, with massive local release of pharmacological mediators at the site of infection, resulting in the expulsion of the parasite from the body. It is still not known why a certain number of people synthesize the large quantities of IgE which cause allergic reactions, but may be elicited as an immune response to foreign antigens and persist because they are normally biologically advantageous. The allergic reactions caused by this IgE may therefore be an unfortunate consequence of maintaining this level of immunity.

The role of IgG in allergic reactions remains controversial. Studies in the human have suggested that IgG antibodies may act as short term sensitizing anaphylactic antibodies (Parish 1970; Bryant et al 1973), and certainly there are IgG receptors on human basophils (Ishizaka et al 1972). Work by Stanworth and Smith (1973) has suggested that this IgG is of the subclass IgG_4, but this is far from proven. The role of IgG as a second anaphylactic antibody thus remains to be elucidated. It has also been suggested that IgG (particularly IgG_4) may act as a blocking antibody, thus preventing

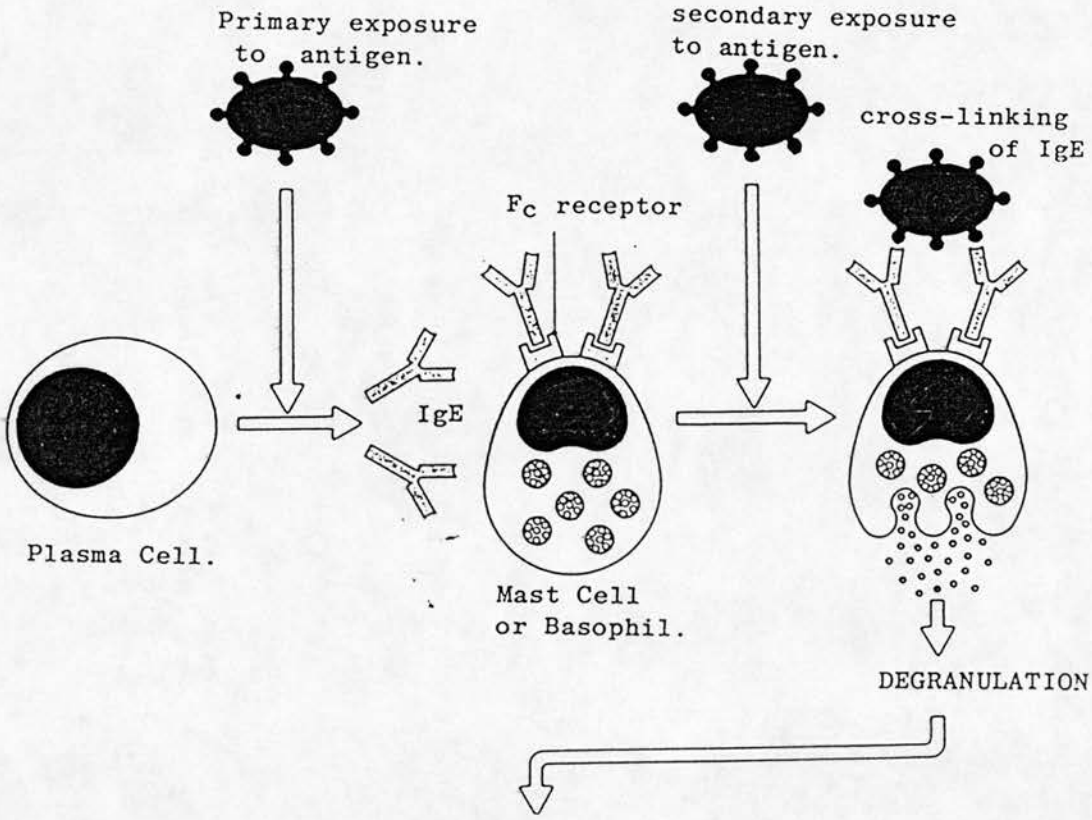


Figure 1: **The Mechanisms of Type 1 Hypersensitivity.**

Primary sensitising exposure to the antigen stimulates the production of IgE-specific antibody. This binds to the F_c receptors on the surface of mast cells or basophils. Secondary and subsequent exposure to the same antigen will cross-link the cell-bound IgE, resulting in the release of the secretory granule contents to produce the effects listed.

Modified from Staines *et al* 1985.

anaphylactic reactions; this may well be the case in certain circumstances, though final proof of this is lacking.

(I). Mast Cells and Basophils.

Mast cells and basophils are central participants in a variety of inflammatory and immunological disorders, particularly in diseases involving immediate hypersensitivity, and share several notable properties:

a. Both have surface receptors with high binding affinity for IgE antibody (Ishizaka et al 1979).

b. Both contain prominent cytoplasmic granules with affinity for certain basic dyes, which appear electron dense by electron microscopy. These granules are the source of potent chemical mediators implicated in a wide spectrum of inflammatory and immunological processes.

c. Exposure of IgE-sensitized mast cells or basophils to appropriate antigen results in the non-cytolytic release of preformed and newly generated chemical mediators from the granule matrix.

d. Exocytotic degranulation of basophils and mast cells may be induced by many immunologically specific and non-specific agents.

Despite these prominent similarities, there are many features which distinguish the two cell types:

a. The nucleus of mast cells is generally large and round. The nuclear chromatin is often aggregated beneath the nuclear membrane, not densely clumped as in basophils and other granulocytes, which causes the irregular lobed characteristic appearance of the nucleus in these cells.

b. Basophils have a short half life of 2-5 days, with only 12 hours in the circulation (Mitchell and Askenase 1983), whereas mast cells are long lived, with a half life of 3-4 months (Padawer 1974).

c. Mast cells are usually larger (10-30μ m diameter) than basophils, which are the smallest of the human granulocytes with a diameter of 10-14μ m (Ackerman and Bellios 1955). The cytoplasmic granules of human basophils display marked size heterogeneity (up to 1.2μ m diameter, over twice the size of human mast cell granules), but are generally fewer and more widely separated than those of mast cells (Parwaresch 1976).

d. The cytoplasmic granules of mast cells stain with greater intensity than those of basophils. This is due to the granular proteoglycan content. Mast cell granules contain heparin, whereas basophil cytoplasmic granules contain a proteoglycan which is less sulphated than heparin, the exact nature of which has yet to be determined.

e. Human mast cells contain more histamine (1-15μ g of histamine per 10^6 cells; Patterson et al 1976), than basophils (1-3μ g of histamine per 10^6 cells; Ishizaka et al 1973).

f. Although both cell types originate in the bone marrow, they are thought not to have a common precursor cell. Basophils, like other polymorphonuclear granulocytes differentiate and mature in the bone marrow, circulate in the blood, and do not normally occur in extravascular sites other than the bone marrow (Dvorak 1978). Mast cells in contrast, are normally distributed throughout the connective tissue, often situated adjacent to blood and lymphatic vessels, and near to or within peripheral nerves. They may be especially abundant near epithelial surfaces such as those of respiratory and gastrointestinal systems and the skin, that are

exposed to environmental antigens (Riley 1959; Brinkman 1968; Trotter and Orr 1974; Feltkamp-Vroom et al 1975; Padawer 1978). Mast cells do not usually circulate in the blood, but are a normal component of the bone marrow and lymph node connective tissues (Spicer et al 1975), and in some species are also abundant in the fibrous capsules of internal organs and in physiological transudates such as peritoneal fluid (Padawer 1978).

(i). Mast Cells.

Both mast cells and basophils are bone marrow derived, but while basophils differentiate and mature in the bone marrow, mast cells are thought to mature outside the bone marrow or circulation, generally at local sites in the connective tissue or serous cavities, from mitotically active precursor cells. Recent studies in mice suggest that the proliferation and differentiation of these precursors are thymus dependent, and are under the influence of local factors including interleukin 3 (Guy-Grand et al 1984). Studies of rodent mast cells have revealed that these precursor cells, which may circulate in the blood (Zuker-Franklin et al 1981), contain few cytoplasmic granules with little or no heparin (Combs et al 1965). As the cells mature, their proliferative capacity is markedly reduced and the number of cytoplasmic granules increases (Combs et al 1965). This is complicated further by recent reports indicating that rodents possess clearly distinguishable populations of mast cells in the connective tissue and in the intestinal mucosa (Enerback 1981; Barrett and Metcalfe 1984).

Morphology.

In humans, as in experimental animals, mature mast cell morphology and size vary somewhat depending on the organ or site of origin,

but in general they appear in tissue sections as round, oval or elongated cells, 10-30μ m in diameter (Trotter and Orr 1974). The nucleus is usually round to oval, with chromatin that becomes progressively more clumped and densely stained with cell maturation. Cytoplasmic granules are round and closely packed, 0.1-0.4μ m in diameter and may have a complex substructure with a unique spiral or crystalline appearance (Trotter and Orr 1974; Padawar 1978). Unlike human basophils, mature human mast cells do not contain electron-dense aggregates of cytoplasmic glycogen, but they do frequently exhibit centrioles and a complex system of uniformly distributed, thin elongated folds and processes on the plasma membrane surface.

(ii). Basophils.

Morphology.

Basophils are derived from the same bone marrow precursors as eosinophils and neutrophils. They normally comprise approximately 0.3% of nucleated cells in the bone marrow, and 0.5-1.0% (20-45 cells/mm^3) of the circulating leucocytes, and are thus the least common human granulocyte. The human basophil has a segmented multilobed nucleus with dense irregular condensations of chromatin along a thin nuclear membrane. The nucleus is quite similar to that of the neutrophil and eosinophil, although the segmentation of the nuclear lobes is less pronounced. The characteristic cytoplasmic granules of the basophil are round to angular, membrane-bound structures. The granule substructure has a distinct crystalline appearance (Parwaresch 1976), and is composed of dense particles in a less dense matrix with occasionally complex membrane structures (Dvorak 1978). A subpopulation of smaller cytoplasmic granules has

been reported in human basophils (Ackerman and Clark 1971; Nichols and Bainton 1973; Hastie 1974). These differ both ultrastructurally and histochemically, and are usually situated between the nuclear lobes. After degranulation, intact electron-dense granules are depleted, and appear as large membrane-bound vacuoles. Numerous cytoplasmic aggregates of glycogen particles are also present with an inconspicuous Golgi apparatus, a few mitochondria, ribosomes, several strands of rough endoplasmic reticulum, and numerous electron-lucent 50-70nm cytoplasmic vesicles. These form a complex vesicular system, which plays a critical role during the degranulation process.

Occurrence.

The involvement of the basophil has been implicated in a wide variety of immune-inflammatory disease states; the most important and relevant to the present study being its involvement in immediate hypersensitivity reactions.

Immediate Hypersensitivity Reactions.

In humans, basophils contain virtually all of the histamine present in blood (Graham et al 1955; Sampson and Archer 1967), and its release in conjunction with a variety of low-molecular-weight mediators in response to antigenic stimuli is clearly established, and is strong evidence for their involvement in immediate hypersensitivity reactions. Allergic atopic diseases have a major immediate hypersensitivity component, and thus the early symptoms of allergic disorders such as allergic rhinitis, asthma, urticaria, anaphylaxis, food and drug allergies, after exposure to allergen, are IgE-mediated events.

The clinical symptoms of allergic disease are diverse, and are determined by various host factors, including:

(i) The organ affected.

(ii) Properties of the allergen involved, previous exposure to the allergen, and its dose and route of administration.

(iii) Class and concentration of circulating tissue-fixed IgE (homocytotropic) antibody.

(iv) Presence and concentration of antigen-specific blocking (non-homocytotrophic) antibody.

Interaction of the allergen with specific IgE antibody causes aggregation of F_c receptors on the cell surface, and results in anaphylactic degranulation with the secretion of granule contents. Immediate hypersensitivity responses involving basophils are systemic in nature, since basophils are normally present only in the circulation or bone marrow. Massive basophil degranulation occurs with generalized systemic release of both preformed and newly synthesized mediators (James and Austen 1964). These orchestrate the inflammatory response that develops, and cause smooth muscle contraction, vascular dilation, vessel permeability, and the recruitment of infiltrating leucocytes. Symptoms include vascular collapse and changes in target organs which frequently include skin, larynx, trachea, oesophagus, eyes and periorbital region.

Delayed-type Hypersensitivity Responses.

Basophils are also thought to play a role in certain delayed hypersensitivity reactions. Most observations of delayed-type immune reactions, in which basophils are actively recruited to

leave the circulation and enter the site of an inflammatory reaction, have been made in the skin of experimental animals; the lesions that develop in such reactions are termed cutaneous basophil hypersensitivity (CBH), (Dvorak et al 1970). Sensitized guinea pigs (immunised by injection of protein antigens without mycobacterial adjuvants) develop a systemic, though tenuous form of delayed-type hypersensitivity (DTH); one of the principle characteristics of these reactions is the striking infiltration of basophils to tissues.

Like classic tuberculin-type DTH, CBH reactions have a delayed time course, becoming apparent at 4-6h, maximal at 18-24h, after skin test. CBH reactions also contain a large mononuclear cell infiltrate, and are in part lymphocyte dependent (Dvorak et al 1970). They are typically flat, non-indurated lesions with little fibrin deposition, whereas classical DTH reactions are characterized by local activation of clotting mechanisms with extensive dermal fibrin deposition, leading to induration, and contain very few basophils.

In addition to cutaneous reactions, basophils also participate to varying extents in delayed-onset immunological reactions elicited in the eye (Friedlander and Dvorak 1977), gut (Askenase et al 1978), subcutis (Dvorak et al 1979a) and peritoneal cavity (Dvorak and Dvorak 1972).

Similar reactions to those of CBH, induced after sensitization by repeated intradermal injections of small quantities of rabbit serum proteins, were found in the skin of human volunteers by Jones and Mote (1934), leading to the name "Jones-Mote" reaction. The relation of these human responses to those of the guinea pig is uncertain, although they differ from classic, tuberculin DTH

responses in the same way as CBH, and one of their principal characteristics is the striking infiltration of basophils (Askenase and Atwood 1976). Nevertheless, basophil accumulation can occur in tuberculin lesions of purified protein derivative (PPD), and other immune reactions of the skin (Dvorak et al 1974; Askenase and Atwood 1976). These include lesions of allergic contact dermatitis (Dvorak and Mihm 1972), and delayed time course late phase responses (DeShazo et al 1979). In humans, basophils are a significant component (up to 5%) of the cells infiltrating sites of skin allograft rejection responses (Dvorak et al 1979b) and detailed study of bullous pemphigoid lesions in various stages of development have revealed infiltration of basophils at these sites (Dvorak et al 1982).

It appears therefore, that in humans, basophil involvement in DTH represents only a part of the complex spectrum of DTH responses, rather than a distinct form of response.

Studies of immune reactions in humans at various sites other than the skin, have resulted in the identification of a role for the basophil in various immune inflammatory reactions in different organ systems.

Nose. Basophils have been demonstrated in nasal secretions (Hastie et al 1979), presumably having migrated through the lamina propria and epithelium. The emigration of basophils into the nasal tissues and secretions occurs after nasal provocation with allergen, or during the appropriate pollen seasons.

Conjunctiva of the eye. One of the morphologic changes evident in vernal conjunctivitis, is the infiltration of basophils to the epithelium of substantia propria (Collin and Allansmith 1977).

Lung. The appearance of basophils in the sputum of patients, during the various phases of bronchial asthma has been reported, and suggests that basophils move from the bloodstream into bronchial tissue during the acute phase of an asthma attack (Kimura et al 1975). It has also been reported that in vitro histamine release can be induced by nuclear components in leucocytes of patients with both chronic bronchitis and non-atopic asthma (Juhl et al 1981), but the relevance of this finding to and the involvement of the basophil in the pathogenesis of this disease is unclear.

Intestine. Examination of diseased tissue from the bowel wall of patients with Crohn's disease has revealed the presence of basophils, which show varying degrees of degranulation (Dvorak et al 1980). The peripheral blood basophils also degranulate in vivo in these patients when the disease is active (Dvorak and Monahan 1982).

Kidney. Basophil involvement has been demonstrated in the inflammatory responses of a variety of different forms of renal disease, including penicillin-induced allergic interstitial nephritis (Colvin et al 1974), and renal allograft rejection (Colvin and Dvorak 1974).

Blood. Elevated levels of circulating peripheral blood basophils

occur in patients with chronic myelogenous leukaemia (CML) (Juhlin 1963a; Juhlin 1963b), and in patients with systemic lupus erythematosus in vivo degranulation of peripheral blood basophils occurs during the acute phase of this disease (Camussi et al 1981).

Cancer. Basophil infiltration has been demonstrated in cancerous tissue as part of the cellular response (Black and Leis 1973), and increased numbers of peripheral blood basophils occur in patients with lung cancer (Anthony 1982). It seems likely therefore that a role for the basophil in the immune response to malignancy exists.

Both basophils and mast cells exhibit phagocytic capability under certain circumstances (Dvorak et al 1972; Padawar 1969), but its importance in the normal function of these cells is unclear. The frequency of basophils in most mammalian species is similar to that in man, although rabbits may have up to 10 times as many. There is a striking inverse relationship between the frequency of basophils and mast cells within different animal species. For example, in animals in which basophils are normally rare or absent, such as rats and mice, tissue mast cells are numerous, and vice versa. In some lower vertebrates, only a single type of metachromatic granule-bearing cell is present, which exists in both the connective tissue, and in the circulation (Stadecker et al 1977). These considerations, together with the biochemical similarities of the two cell types supports the widespread presumption that these cells supplement each other, and have similar or even identical functions.

II. Stimuli for Basophil/Mast Cell Activation.

The process of basophil or mast cell degranulation is a secretory event, and may occur in response to a long and growing list of immunologically specific and non-specific agents. Sensitivity to many of these stimuli varies strikingly according to species and cell type, but only the immunologic stimuli are relevant to the study of human allergic reactions.

(i). Immunological Stimuli.

IgE Receptor Mediated.

The activation of mast cells and basophils depends on the cross bridging of the F_c receptors, to which IgE antibodies are bound, with the F_{ab} portion of the molecules available for interaction with specific antigen (Figure 1). Thus activation of the cell is dependent on the antigenic specificity of the IgE antibody. This process requires bridging of very few (1%) of the IgE molecules on the cell surface.

In addition to specific antigen, cross-linking of cell bound IgE may also be induced by heterologous (for example, rabbit or goat) antisera to IgE or to immunoglobulin light chain (κ or λ). Anti-IgE-receptor mediated antibody may also cause this event.

Protein A from Staphylococcus aureus, and mitogens such as Concanavalin A also activate cells by cross-linking the carbohydrates of the IgE molecules (Siraganian 1976).

IgG Receptor Mediated.

Mast cells and basophils have F_c receptors distinct from the IgE receptor, that bind IgG molecules (Ishizaka et al 1970). Since the binding of IgG molecules is of much lower affinity, this is a less efficient system than activation through the IgE receptor.

(ii). Non-immunological Stimuli.

Complement fragments.

Mast cells and basophils may be activated by two major complement-derived anaphylatoxins $C5_a$ and $C3_a$, via distinct receptors which are different from the immunoglobulin receptors (Siraganian and Hook 1976a). $C5_a$ is a more potent anaphylatoxin than $C3_a$, and $C4_a$ may weakly interact with $C3_a$ receptors. $C3_b$ receptors have been demonstrated on human basophils (Thomas and Lichtenstein 1979) and may induce cell activation, but these are not present on mast cells.

Lymphokines.

These lymphocyte-derived mediators activate basophils directly (Thueson et al 1979) or indirectly by modulating the cell activation process induced by other secretagogues, such as viral and immune interferon (Ida et al 1977).

Basic peptides.

Formyl methionine tripeptides activate human basophils via specific receptors, but have no effect on rat mast cells. These basic peptides are chemotactic for a number of cell types, and simulate various bacterial chemotactic factors (Siraganian and Hook 1977).

Eosinophil granule major basic protein.

This basic protein, which is rich in arginine, is the main constituent of eosinophil granules, and causes activation of human basophils and mast cells (O'Donnell et al 1983).

Other compounds.

An ever increasing list of other compounds can activate mast cells and basophils by various mechanisms. For example, ionophores are lipophilic molecules that insert themselves into cell membranes and transport Ca^{++} and Mg^{++} ions across the membrane, thereby increasing intracellular calcium and magnesium concentrations (Foreman et al 1973). Mast cells are activated by various polycationic substances such as compound 48/80, polymyxin B, polylysine and polyarginine. Other compounds which cause activation include morphine, tubocurarine, protamine, ATP, dextran (between $4x10^5$ and $2x10^6$ molecular weight), chymotrypsin, mellitin, mast cell degranulating peptide from cobra venom, and a cationic lysozymal granule protein from neutrophils (Siraganian 1985).

(III). Mechanisms of Mediator Release.

The activation of basophils and mast cells is initiated by interaction of antigen with specific IgE on the cell surface, and leads to a complex series of biochemical events which culminate in the secretion of mediators from the cell (Levine 1966). This process is generally termed anaphylactic degranulation. The association between degranulation and the release of histamine in rat mast cells was first described by Riley in 1953. Later, Fawcett (1955) confirmed this, and demonstrated the non-cytolytic nature of the degranulation process. Thon and Uvnas (1967) proposed a two stage process for degranulation. This involves exposure of the cytoplasmic granules to the cell exterior, followed by release of histamine by a process of cationic exchange upon contact of the granule matrix with the extracellular fluid. Classic experiments of Rohlich et al (1971) provided conclusive evidence that

degranulation is a secretory (or exocytotic) event that is non-cytotoxic, in which the membranes surrounding individual secretory granules fuse with the plasma membrane of the cell, and in some cases, also with each other.

Most of the events associated with IgE-mediated degranulation are common to both basophils and mast cells, although certain differences have been noted (Lichtenstein 1968; Lichtenstein et al 1973). A general outline of these events is therefore presented, highlighting any differences between basophils and mast cells where appropriate.

A speculative scheme of the events leading to mediator release has been proposed (Siraganian 1983), and these are summarized in Figure 2.

(i). Biochemical Events of Mediator Release.

One of the earliest events of cell activation is thought to involve the activation of a membrane-associated proteolytic enzyme, which is likely to be a serine esterase closely associated with the receptor for IgE (Becker and Henson 1973). IgE receptor aggregation is thought to activate methyl transferase enzymes, resulting in membrane phospholipid methylation, and the formation of phosphatidylcholine. This in turn causes local changes in transmembrane potential, and opens up Ca^{++} channels. The increased intracellular Ca^{++} concentration activates the enzyme phospholipase A_2, resulting in the formation of arachidonic acid and lysolecithin by cleaving phosphatidylcholine or phosphatidylinositol. The lysolecithin is thought to promote membrane fusion, and arachidonic acid is metabolized by either the cyclooxygenase or lipooxygenase pathway, leading to the production of prostaglandins (predominantly

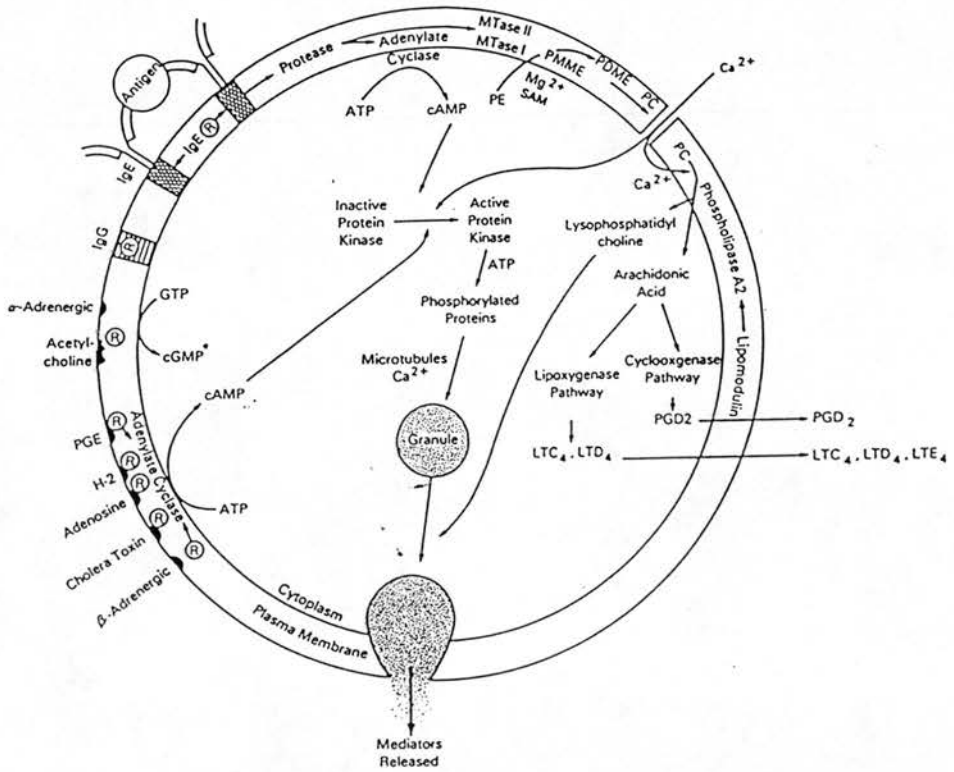


Figure 2: **Proposed Scheme of Events Leading to the Release of Mediators from Mast Cells and Basophils.**

Abbreviations are as follows:

R = Receptor

MTase = Methyl transferase

SAM = S-adenosylmethionine

PE = Phosphatidyl ethanolamine

PMME = Phosphatidyl-N-monomethyl ethanolamine

PC = Phosphatidylcholine

Modified from Siraganian 1985.

PGD_2 in mast cells) and leucotrienes (such as SRS-A) which are discussed later, in section (IV) of this Chapter. These early events also cause the characteristic alterations in intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP) by activation of adenylate cyclase. Increased concentrations of intracellular cAMP result, and together with the increased intracellular Ca^{++} and cGMP levels, they regulate the events that follow. During this step, phosphorylation of proteins is thought to occur, leading to microtubular aggregation, exposing granules to the extracellular space, and resulting in the release of mediators.

The process of mediator release is rapid, has an absolute requirement for the presence of Ca^{++} in the medium and is temperature-dependent with optimal release occurring at 37°C (Siraganian 1985). Metabolic energy is also an essential requirement at multiple steps in the release process, and is thought to be obtained from either aerobic or anaerobic glycolysis, although experiments using a number of cell systems have shown that other cells may utilize oxidative phosphorylation (Johansen 1980).

Calcium Requirement.

In normal cell systems, the low intracellular Ca^{++} concentrations (0.1μ m in cytoplasm) compared with the extracellular Ca^{++} (2mM), are maintained by an energy-dependent pump which moves Ca^{++} out of the cell. Calcium influx into the cell is thought to be the intracellular signal for the initiation of cellular activation, and is therefore essential for mediator release (Rubin 1974). This hypothesis is based on the following observations:

a. Increasing intracellular Ca^{++} levels by Ca^{++} ionophore A23187 which transports Ca^{++} into the cell, microinjection, fusion with

Ca^{++}-containing vesicles, or permeabilization of the cell membrane, all activate the release process (Foreman et al 1973).

b. The release process is inhibited by the removal of Ca^{++} stores, with for example Ca^{++} chelators such as EDTA.

c. It has been demonstrated that the movement of $^{45}Ca^{++}$ into the cell occurs prior to the release of mediators (Foreman et al 1973; Ishizaka et al 1983).

Intracellular Ca^{++} is thought to regulate a large number of enzyme systems, including phospholipase A_2 and microtubule assembly, by binding to calmodulin. This is a ubiquitous protein, with a molecular weight of 17,000 and four binding sites for Ca^{++}. The binding of Ca^{++} to any one of the four binding sites mediates a conformational change, which results in the association of this complex with enzymes.

Phospholipid Methylation.

The process of phospholipid methylation in the early events of degranulation is thought to function in signal transduction across the cell surface (Hirata and Alexrod 1980). There are two methyltransferases in the cell membrane.

a. Methyltransferase I, which faces the cytoplasmic surface, and transfers one methyl group from S-adenosylmethionine (SAM), a high-energy methyl donor, to phosphatidyl ethanolamine (PE) (Figure 3), resulting in the formation of phosphatidyl-N-monomethyl ethanolamine (PMME).

b. Methyltransferase II, which faces the extracellular space, then adds a further two methyl groups resulting in the formation of phosphatidylcholine (Figure 3).

Only IgE-mediated cell activation requires this phospholipid

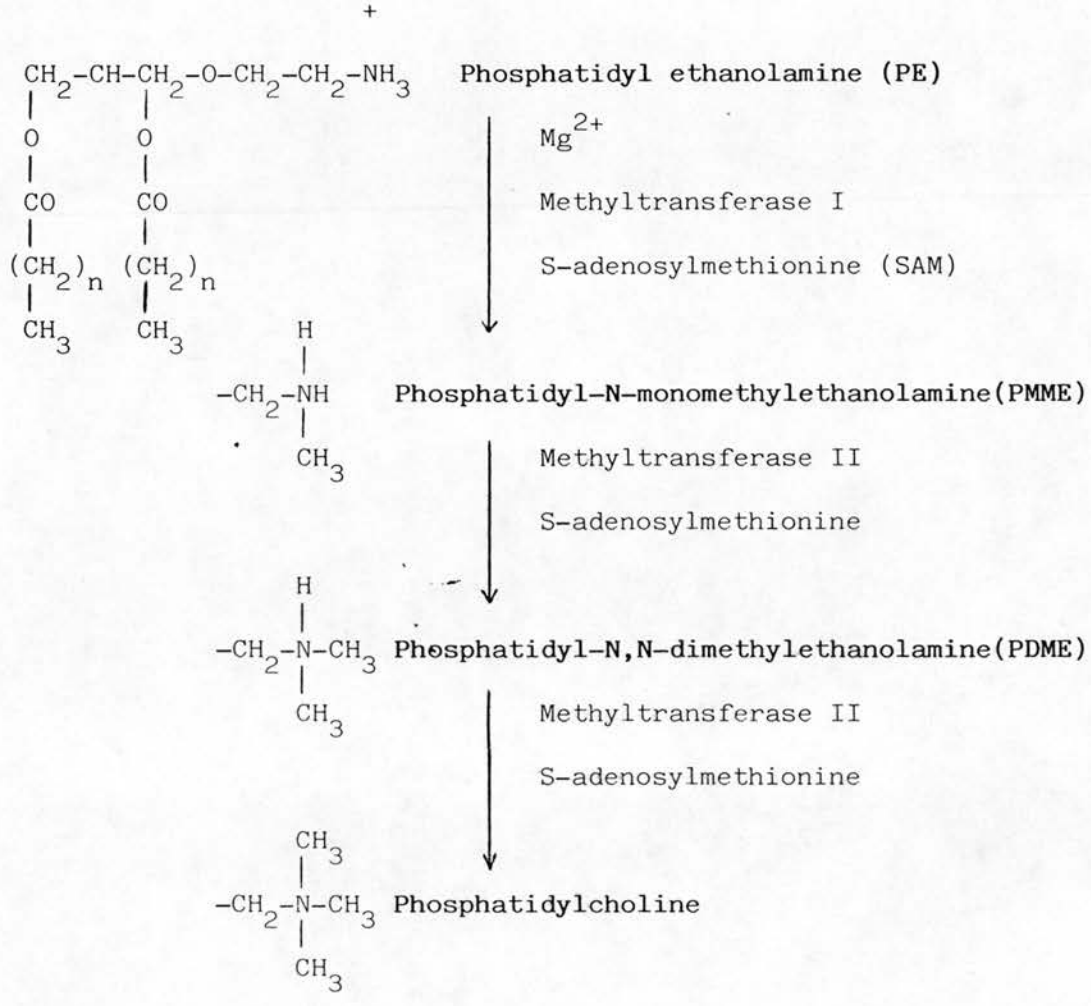


Figure 3: **Biochemical Pathway for Phospholipid Methylation.**

methylation, since inhibitors of methylation prevent IgE receptor-mediated cell activation, but have no effect on the activation of cells by, for example, compound 48/80, the calcium ionophore A23187, formyl methionine tripeptides, or complement fragments (Morita et al 1981; Morita and Siraganian 1981). The activation of cAMP and methylation enzymes affect each other, and have a complex relationship. Inhibitors which elevate intracellular cAMP, also inhibit methylation, though specific inhibitors of methylation suppress the early increase in cAMP levels (Ishizaka et al 1981). Nevertheless, the initial IgE-mediated activation sequence appears to require activation of a membrane enzyme to facilitate phospholipid methylation, and increase intracellular levels of cAMP and Ca^{++} (Ishizaka et al 1984).

Cyclic Nucleotides.

Activation of cells by IgE-mediated mechanisms results in an increase in intracellular (cAMP) levels, which is a very rapid process, and parallels phospholipid methylation (Ishizaka et al 1981). A large number of substances may alter intracellular levels of cAMP, including ß -adrenergic agents, some prostaglandins (for example PGE_2), histamine via H_2 receptors, cholera toxin and adenosine. The interaction of these ligands with specific cell-surface receptors results in the activation of the membrane-associated enzyme adenylate cyclase, and therefore the formation of cAMP. The exact role of cAMP in mediator release is not known; a transient monophasic rise in intracellular cAMP is necessary for cellular activation, although high levels of intracellular cAMP inhibit mediator release. The intracellular effects of cAMP are mediated by the cytoplasmic cAMP-dependent

protein kinases, which are involved in the phosphorylation of contractile or cytoskeletal proteins, such as myosin light-chain subunits, microtubule-associated protein, or tubulin subunits. This process is thought to modulate the triggering of mediator release, and inhibit secretion (Winslow and Austen 1982). Phosphorylation of myosin light-chain kinase inhibits contraction and movement of granules to the cell surface, and mobilization of Ca^{++} from intracellular pools may be inhibited by phosphorylation of a protein which binds Ca^{++} (Winslow and Austen 1984). Increased levels of intracellular cGMP consequent on exposure to cholinergic agents such as acetylcholine or carbachol, also enhance IgE-mediated secretion in mast cells. These agents however, have no effect on human basophils.

(IV). Pharmacological Mediators of Mast Cells and Basophils.

The mediators that are secreted from mast cells and basophils when activated in immediate type hypersensitivity reactions are either preformed or are newly generated on cellular activation. The content of these substances in human basophils and mast cells is slightly different, and a further level of complexity exists in the content of the two mast cell subsets in rodents. The major differences are outlined in Table 1, and the substances involved are described below.

(i). Preformed mediators of secretory granules.

Biogenic Amines.

Histamine.

Histamine (β -imidazolylethylamine) is common to mast cells and basophils of most animal species, with the exception of fish and

Table 1: **Mediators of Mast Cells and Basophils.**

MEDIATOR	**RAT**			**HUMAN**
Preformed Mediators	Connective tissue mast cell	Mucosal mast cell	Mast cell	Basophil
Biogenic amine	Histamine	Histamine	Histamine	Histamine
Major proteoglycan	Heparin	Not determined	Heparin	Not determined
Predominant neutral protease(s)	Chymase (RMCPI) Carboxypeptidase A	RMCP II	Tryptase	Kallikrein (Bradykinin activator)
Newly Generated Mediators				
Arachidonic acid metabolites	PGD_2	Not determined	LTC_4 PGD_2	-
Platelet activating factor	-	-	+	-

amphibians (Chiu and Lagunoff 1972). The major source of tissue histamine was found to be the mast cell (Riley and West 1953), and the major source of humoral histamine was attributed to the basophil fraction by Graham et al (1955). In rabbits however, although basophils are relatively frequent, they contain only about 25% of blood histamine, the remainder being associated with the platelets (Benveniste et al 1973). Rodent connective tissue mast cells contain 10-30μ g, and human mast cells 1-15μ g of histamine per 10^6 cells (Schwartz and Austen 1981), whereas normal human basophils contain only 1-3μ g of histamine per 10^6 cells (Ishizaka et al 1983; MacGlashan and Lichtenstein 1980).

The histamine present within basophils and mast cells is thought to be confined largely to their specific cytoplasmic granules, as revealed by cell disruption studies (Pruzansky and Patterson 1967), and is released by immunological reactions that result in degranulation of the cell (Lichtenstein 1968). These considerations have allowed for the use of histamine release from these cells as the major biochemical marker for cellular activation and degranulation.

The synthesis and metabolism of histamine in these cells is described and discussed in detail in Chapter 2:2.

Serotonin.

5-hydroxytryptamine [3-(β -aminoethyl)-5-hydroxyindole] or serotonin is formed from tryptophan, by the sequential action of tryptophan-5- hydroxylase and aromatic L-amino acid decarboxylase, producing 5-hydroxytryptophan and 5-hydroxytryptamine respectively (Lagunoff and Benditt 1959). Serotonin was first purified and crystallized from human serum by Rapport in 1949, and chemically

synthesized by Hamlin and Fischer in 1951. In humans, serotonin is absent from both basophils and mast cells, but is contained in platelets and tissue of the central nervous system. It is concentrated in the hypothalamic region of the brain, where it is localized in granular synaptic vesicles in nerve endings. In rodents, serotonin is absent from mucosal mast cells and basophils, but is contained in connective tissue mast cells at concentrations of about 1μ g of serotonin per 10^6 cells (Moran et al 1962). It is packaged in the secretory granules, associated with serotonin binding proteins, and is released in parallel with histamine, when it is sequentially degraded to 5-hydroxyindoleacetaldehyde and 5-hydroxyindoleacetic acid by monoamine oxidase and aldehyde dehydrogenase respectively (Udenfriends et al 1956).

In rats, serotonin enhances peripheral vasopermeability, and in mice is involved in delayed hypersensitivity reactions. In humans, serotonin raises body temperature by a hypothalamic mechanism, enhances vasopermeability and causes contraction of many smooth muscle systems.

Dopamine.

Dopamine is a catecholamine intermediate in the synthesis of noradrenaline and adrenaline, and is normally found in mammals concentrated in the corpus striatum region of the brain, where it functions as an inhibitory neurotransmitter. Dopamine is a further biogenic amine found in bovine mast cells (Jenkinson et al 1970).

Proteoglycan.

Proteoglycans are complex macromolecules composed of glycosaminoglycans (GAGs) associated with a protein core by means

of covalent bonds, and are responsible for the characteristic granule metachromatic staining of basophils and mast cells with basic dyes. The structural differences and properties of the different types of proteoglycan are detailed in Chapter 2:1.

The predominant proteoglycan in secretory granules varies with cell type and species. Human mast cells, rat and mouse connective tissue mast cells and rat mast cells grown from embryonic fibroblast monolayers contain heparin (Metcalfe et al 1979; Metcalfe et al 1980; Bland et al 1982), whereas bone marrow-derived mouse mast cells have been shown to contain chondroitin sulphate E (Razin et al 1982). The GAGs of bone marrow-derived rat mast cells and normal human basophils are less sulphated than heparin, and have yet to be identified unequivocally. The nature and content of GAG in the various cell types is discussed in Chapters 3 and 4.

Intracellularly, the highly ionic proteoglycans form the matrix of the secretory granule, ionically bound to cationic proteins including some proteases. These proteoglycan-protein complexes are in turn attached by ionic linkages to many of the preformed mediators such as histamine (and where present, serotonin), Zn^{++} and other pharmacologically active granule constituents such as eosinophil chemotactic factor (ECF-A) (Thon and Uvnas 1966; Uvnas et al 1970). These complexes are packed into a small nuclear volume to allow the formation of the macromolecular complex of the secretory granule (Schwartz et al 1981a). On degranulation, ion exchange mechanisms result in the release of these loosely bound small molecules from the secretory granules (Aborg and Uvnas 1968), and it has also been shown that basophil and mast cell proteoglycans are themselves released from the cell during this process (Yurt et al 1977). Heparin is tightly bound to tryptase in

human mast cells (Schwartz et al 1981a), and chymase and carboxypeptidase A in rat mast cells (Schwartz et al 1981b; Schwartz et al 1982). These complexes are thought to regulate various secretory granule enzyme systems and facilitate the uptake of histamine into the secretory granule against a concentration gradient (Uvnas et al 1970).

Neutral Proteases.

Neutral proteases are the third major component of human and rat serosal mast cell secretory granules. Gomori (1953) demonstrated neutral protease activity in rat mast cells, and later Benditt and Arase (1959) showed its chymotrypsin rather than trypsin substrate specificity, by cleavage of the acetylethyl esters of aromatic and not basic amino acids. By contrast, neutral proteases of human mast cells have trypsin-like activity and cleave N-α -benzoyl-DL-arginine -β -naphthylamide hydrochloride (Glenner and Cohen 1960). Mast cell proteases are strongly positively charged which has important implications for interaction of these enzymes with intragranular proteoglycans.

Rodent Mast Cell Neutral Proteases.

Chymase (RMCP I) and carboxypeptidase A are present in the secretory granules of rat serosal mast cells; chymase has been localized to the granules of mast cells from rat skin, muscle, lung parenchyma and serosal cavities, and carboxypeptidase A is found in peritoneal mast cells and in skeletal muscle where the mast cell is the most likely source (Woodbury et al 1978a; Everitt and Neurath 1980; Bodwell and Meyer 1981). By contrast, mast cells from gastrointestinal and bronchial mucosa contain a chymotrypsin-like

protease referred to as rat mast cell protease II (RMCP II) (Woodbury et al 1978b). These neutral proteases are complexed with proteoglycan in the secretory granules, and are released along with histamine and ß -hexosaminidase during IgE-mediated degranulation.

Human Mast Cell Neutral Proteases.

Tryptase is an endopeptidase that cleaves peptide and ester bonds on the carboxyl side of basic amino acids (Schwartz et al 1981a). Like the rat mast cell neutral proteases, tryptase is the principal protease of the secretory granules of human pulmonary mast cells, where it resides as a complex with proteoglycan. It is released in parallel with IgE-mediated release of histamine and can also serve as a marker for mast cell activation (Schwartz et al 1987). Tryptase accounts for about 23% of total mast cell protein, a similar level to that of neutral protease in rat mast cells, and is present in concentrations of about 12µ g per 10^6 cells (Schwartz 1986).

A second protease, a serine esterase is found in the secretory granules of human mast cells of the skin and interstitial submucosa, exhibits a cleavage pattern similar to that of pancreatic chymotrypsin (Irani et al 1986). Calculations of potential amounts of this enzyme in cutaneous mast cells range from 0.1-1.0µ g per 10^6 mast cells, approximately 1-10% of the amount of tryptase present in human pulmonary mast cells.

Human Basophil Neutral Proteases.

A bradykinin-generating enzyme (BK-A) has been found in human basophils, and is a kallikrein-like endopeptidase which generates bradykinin from high molecular weight kininogen, and it is released along with histamine by IgE-mediated activation (Newball et al

1979).

The exact role of the neutral proteases of mast cells and basophils is difficult to define, since their natural protein substrates have yet to be determined. The possible biological effects of these enzymes may be as follows:

a. Degradation of blood-vessel basement membrane, with resultant increased vascular permeability.

b. Degradation of connective tissue components, such as the peptide core of proteoglycans, allowing the influx of secondary inflammatory cells.

c. Degradation of debris and activation of growth factors to promote wound healing.

Although plasma proteins, including angiotensin I and C_3 have been shown to be substrates for these proteases in vitro, the physiological relevance of these observations remain to be demonstrated.

Preformed Chemotactic Factors.

Mast cell degranulation in vivo results in the influx of secondary cells such as eosinophils, neutrophils and lymphocytes, recruited to the sites of immediate hypersensitivity reactions by mast cell-derived chemotactic factors. Studies to identify factors selective for eosinophils revealed a group of preformed intragranular peptides that were collectively termed 'eosinophil chemotactic factors of anaphylaxis' (ECF-A) and were first described by Kay et al (1971). These exist in low and intermediate molecular weight forms (Goetzl and Austen 1976). Two acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu augment the number of functional C_{3b} receptors on human eosinophils, thereby

facilitating immune adherence and phagocytosis, and are active in concentrations as low as 10^{-8}M (Goetzl and Austen 1976). Exposure to these tetrapeptides causes subsequent deactivation of any further eosinophil chemotactic response.

Two other chemotactic peptides of presumptive mast cell origin are termed eosinophil chemotactic oligopeptide, with a molecular weight of 1200-2500 which preferentially attracts eosinophils in vitro (Austen et al 1976), and high molecular weight neutrophil chemotactic factor of anaphylaxis (HMW-NCF), which has also been described in human basophils (Lewis et al 1974), human lung fragments (Austen et al 1976), and human serum (Atkins et al 1977).

Acid Hydrolases,

A number of acid hydrolases (listed below) have been identified in human and rat mast cell secretory granules, which perhaps defines this organelle as a modified primary lysosome. These enzymes are thought to have mainly extracellular functions, but may also be involved in the degradation of ingested GAGs, glycoproteins and complex glycolipids.

ß -Hexosaminidase: This is an acid exoglycosidase found in both rat and human mast cells, most of which is localized to the secretory granules.

ß -Glucuronidase: This is also an acid exoglycosidase, localized to both rat and human mast cell secretory granules.

ß -D-Galactosidase: This enzyme has been detected in the secretory granules of purified rat serosal mast cells.

Arylsulphatases: Lysosomal arylsulphatases A and B catalyse the hydrolysis of aromatic sulphate esters. Rat mast cells contain approximately equal quantities of arylsulphatases A and B, although only the A subtype is localized to the secretory granule. Human pulmonary mast cells contain these enzymes, although the distribution of the subtypes has not yet been determined.

Oxidative enzymes.

Superoxide dismutase and peroxidase enzymes have been detected in the secretory granules of rat mast cells. Peroxidase is required in the conversion of PGG_2 to PGH_2 in arachidonic acid metabolism, it is capable of destroying leukotriene C_4, D_4 and E_4 activity, and may provide mast cells with cytotoxic capabilities (Henderson et al 1981).

(ii) Newly Generated Mediators of Mast Cells and Basophils.

Whereas the preformed mediators are stored in the secretory granules and can be extracted from quiescent mast cells or basophils, membrane-derived lipid mediators are not formed until after the cells have been activated. The lipid mediators that have been identified include metabolites from the oxidative metabolism of arachidonic acid, and the phospholipid derivative platelet activating factor (PAF).

Arachidonic Acid Metabolism.

After mast cell and basophil activation, arachidonic acid is made available from membrane phospholipids by phospholipase A_2, or by the sequential action of phospholipase C and diacylglycerol lipase

(Figure 4). Arachidonic acid is then processed by one of two separate enzymatic pathways: the cyclooxygenase (prostaglandin synthetase) pathway, which leads to the production of prostaglandins and thromboxanes, or one of the lipooxygenase pathways leading to the production of n-hydroxylipids and dihydroxy fatty acids, and to the sulphidopeptide leucotrienes C_4, D_4 and E_4 (which collectively constitute the substance formerly known as the slow reacting substance of anaphylaxis or SRS-A) (Figure 4).

Different cell types generate distinct arachidonic acid metabolites. In vitro activation experiments have revealed that purified rat and human lung mast cells generate PGD_2 as the predominant arachidonic acid metabolite, whereas only human mast cells generate products of 5-lipooxygenase, including 5-HETE, and the sulphidopeptide leukotrienes (Patterson et al 1976; Lewis et al 1981). Basophils synthesize only the sulphidopeptide leukotrienes (MacGlashan et al 1983).

Prostaglandins.

Arachidonic acid metabolism by the cyclooxygenase pathway leads predominantly to the formation of prostaglandin D_2 by a series of membrane-associated enzyme events (Figure 4). A membrane-associated cyclooxygenase converts arachidonic acid to prostaglandin G_2 (PGG_2) which in turn is converted to PGH_2 by a peroxidase enzyme. Both PGG_2 and PGH_2 are unstable intermediate molecules and are rapidly converted to PGD_2 by endoperoxide-D-isomerase, thromboxane A_2, heptadecatrienoic acid (HHT) or another prostaglandin (Figure 4), which varies with cell type according to the availability of specific enzymes. For example, both rat and human mast cell activation largely generates PGD_2 with lesser amounts of

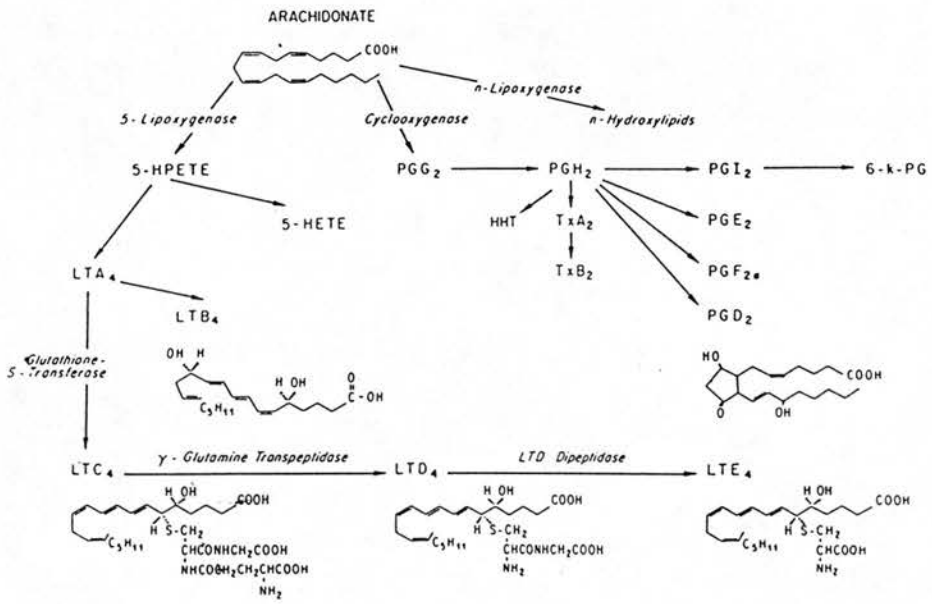


Figure 4: **Biochemical Pathway for the Metabolism of Arachidonic Acid.**

Modified from Schwartz and Austen 1984.

thromboxane A_2 and PGF_2, which is a reduction product of PGH_2 (Lewis et al 1982), whereas human basophils do not synthesize this mediator, although rat basophilic leukaemia cells produce some PGD_2 (MacGlashan et al 1983).

The addition of PGD_2 or inhibitors of the cyclooxygenase pathway (for example, indomethacin) to human mast cells has little or no effect on histamine release (Lewis et al 1982; Lichtenstein 1982), although PGD_2 is thought to enhance immunological release of histamine from human basophils (Lichtenstein 1982). Nevertheless, it is generally believed that the products of this pathway are not involved in the secretory process.

PGD_2 is an important potent mediator of anaphylaxis, and its biological effects in tissues are extensive. Very small amounts of PGD_2 injected into human skin causes increased vasodilation and vasopermeability resulting in an erythematous wheal-and-flare reaction similar to that seen with histamine, which persists for as long as 2h, accompanied by leucocyte infiltration. Inhaled PGD_2 produces bronchoconstriction with a potency about 10 times greater than that of histamine (Smith et al 1980).

Leukotrienes.

The products of the 5-lipooxygenase pathway are the leukotrienes (Figure 4). The 5-lipooxygenase enzyme forms 5-L-hydroperoxy-6-trans-8,11,14- cis-eicosatetraenoic acid (5-HPETE), which is either spontaneously hydrolysed to 5-L-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) or converted enzymatically to the unstable epoxide 5,6-oxide-7, 9-trans-11,14-cis-eicosatetraenoic acid (LTA_4). The LTA_4 either forms LTB_4 or is conjugated with

glutathione to form LTC_4, the first of the SRS-As. Subsequently, two additional SRS-As are generated by the sequential removal of glutathione to yield LTD_4 and of glycine to yield LTE_4 (Figure 4). Inhibitors of arachidonic acid metabolism block histamine release, whereas diverting arachidonic acid to the lipooxygenase pathway (by selective inhibition of the cyclooxygenase pathway, with indomethacin or aspirin) enhances histamine release (Marone et al 1979). These considerations suggest a possible role of a product from the lipooxygenase pathway in cell secretion.

Leukotriene C_4, D_4 and E_4 all elicit erythema and wheal formation when injected into human skin, and inhalation causes bronchospasm and contraction of airway smooth muscle in vivo and in vitro. Antihistamines have no effect on this activity, and a role for these substances as mediators of bronchial asthma is therefore unlikely. These sulphidopeptide leukotrienes are about 30 (LTE_4) to 1000 (LTC_4 and LTD_4) times more potent contractile factors than histamine (Adelroth et al 1986), and are known to cause constriction of coronary arteries. These considerations suggest that these substances contribute to the hypotensive as well as the peripheral airway manifestations of systemic anaphylaxis, but the true relevance of the leukotrienes in immediate hypersensitivity has yet to be defined.

Platelet Activating Factor (PAF).

PAF (1-alkyl-2-acetyl-glyceryl-3-phosphorylchlorine) is produced by mast cells, neutrophils, monocytes and platelets, but not human basophils (Schleimer et al 1986). Although initially PAF was recognized because of its ability to aggregate platelets, it is now known to have potent effects on many other cells including mast

cells, where IgE-mediated release of PAF implicates it as an important mediator of immediate hypersensitivity reactions. The bronchoconstricting properties of PAF have not been tested in humans, although it causes a wheal-and-flare response when injected into the skin, and is about 1000 times more potent than histamine. PAF is a powerful chemotactic agent for polymorphonuclear leucocytes, and thus may be involved in the recruitment of secondary cells to sites of mast cell activation (Schwartz and Austen 1984).

(V). Possible Role for the Basophil.

Despite the vast amount of research on mast cells and basophils in recent years, the exact physiological function or functions of these cells remain poorly understood. It is generally accepted that their role is related to their ability to bind IgE molecules and release mediators contained within their cytoplasmic granules, and is not restricted to hypersensitivity reactions. The functions of mast cells and basophils are thought to be complementary due to their close association with IgE antibody, the most obvious being in the immune defence reaction to parasitic infection (Ogilvie and Jones 1971). During parasitic infection, mast cells or basophils could be activated by parasite-specific IgE, and mediators released leading to eventual parasite expulsion. The majority of parasitic infections are coupled with increased IgE synthesis. These parasites are often large organisms compared to other infectious agents, and therefore defences offered by macrophage and lymphocytes are somewhat inadequate. The IgE sensitized mast cells and basophils may therefore serve to back up these primary defence mechanisms.

Mast cells play a defensive role in local inflammatory reactions, which lead to heightened accessibility of antibodies and complement within the inflamed area. Infiltration and accumulation of various types of immunologically reactive cells (including basophils) results in the activated tissue. Together, these humoral factors and cells form a system of far superior defence power than could have been achieved without inflammatory reactions. Padawer (1978) suggested that basophil attraction to sites of mast cell activation, to release mediators upon interaction with extracellular antigen, causes oedema, vasodilation and increased vascular permeability, accompanied by the influx of IgG, IgM and unbound IgE. This unbound IgE may serve to initiate a second response, interacting with mast cells and leading to the further involvement of antibody and inflammatory reactive cells.

In most parts of the industrialized world, parasitic diseases are rare, yet 10-15% of the population have elevated IgE antibodies directed against various antigens such as pollen, house dust mite, drugs, foods or insect venoms. The participation of the IgE/mast cell/basophil system in these individuals may be due to a failure to limit their proper immune response, coincidentally leading to anaphylactic reactions and the symptoms of allergic disease.

Aims of Study.

The aims of the present major study were as follows:

a. To develop further and refine the sensitive competitive binding assay of Dawes and Pepper (1982), for the measurement of highly sulphated GAGs.

b. To determine the source and exact biochemical nature of the GAG in whole blood, plasma and tissue extracts.

c. To study the conditions of release of this GAG from human basophils in response to antigenic challenge in vitro, using basophil enriched fractions of whole blood from both atopic and non-atopic individuals. Since the release of histamine from basophils is widely used in the study of allergy, and histamine is associated with GAG within the secretory granules, release of the GAG during this response was studied in relation to that of histamine.

d. To study the involvement of GAG in the process of systemic anaphylaxis in vivo, using both human and animal models; patients receiving platelet transfusions and preparations of intravenous immunoglobulin, and Nippostrongylus brasiliensis-infected rats challenged intravenously with worm antigen.

e. To measure numbers of circulating basophils in healthy and atopic individuals, using a new and highly sensitive method of cell counting- the Technicon H-6000, in an attempt to confirm that the basophil has an important role to play in allergic disease.

f. To study the circulating levels of GAG in human plasma and tissues, to answer a number of fundamental questions with regard to its physiological importance. For example, does this circulating plasma GAG level vary with time in individuals, and is plasma GAG concentration related to individual basophil count.

Chapter 2.

(2:1). Measurement of Glycosaminoglycans.

(1). Introduction.

Glycosaminoglycans (GAGs) constitute a class of polysaccharides often associated with protein by means of covalent bonds, to form proteoglycans. GAG side chains attach to a peptide core via the linkage region:

(Disaccharide repeats)-GlucUA β 1-3 Gal β 1-3 Gal β 1-4 Xyl-Serine,

-common to most O-linked GAGs (Grebner et al 1966). There are no formal criteria for identifying GAGs, but are all negatively charged polymers made up almost entirely from repeating disaccharide units; they consist of a carbohydrate backbone containing alternating sequences of uronic acid and hexosamine residues, the only exception being keratan sulphate in which galactose replaces the uronose residue. The hexosamine residues can be:

a. **N-acetylglucosamine,** in heparin, heparan sulphate and hyaluronate.

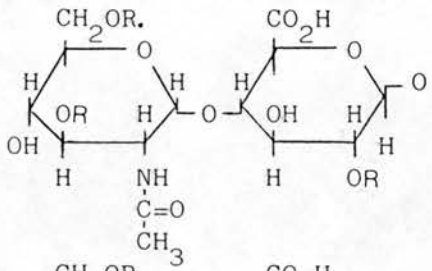
or b. **N-acetylgalactosamine,** in chondroitin sulphates and dermatan sulphate.

All GAGs (with the exception of hyaluronate) contain ester sulphate groups at the C_4 or C_6 position of the acetyl hexosamine residues. The residues of heparin and heparan sulphate may be N-acetylated or N-sulphated, a feature unique to these two polysaccharides (Lindahl and Hook 1978).

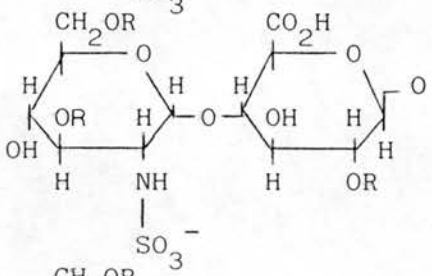
Heparin.

In heparin the disaccharide units are composed of D-glucuronic or L-iduronic acid in ß 1-4 linkage to glucosamine (Helting and Lindahl 1972). Figure A shows three forms which may exist; an N-acetylated type **(i)**, and two N-sulphated types **(ii)** and **(iii)**, and in each example R may be SO_3^- or H. In "full-fledged" heparin, iduronic acid is by far the most abundant uronic acid component, and accounts for 70-90% of the total uronic acid (Roden 1980).

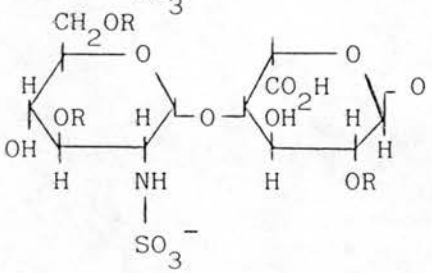
Figure A.



(i) GlcNAc 1-4GlcUAß 1-4.



(ii) $GlcNSO_3$ 1-4GlcUAß 1-4.



(iii) $GlcNSO_3$ 1-4GlcUA 1-4.

One carboxyl group, and one to four sulphates may be present per disaccharide unit. These sulphate residues are substituted at the nitrogen of glucosamine, the C-3 and C-6 hydroxyl groups of glucosamine, and the C-2-hydroxyl group of iduronic acid. The protein core of heparin isolated from rodent skin tissue (Robinson et al 1978) and serosal mast cells (Metcalfe et al 1980) contains

alternating serine and glycine amino acid units. Every second or third serine is linked to a glucosaminoglycan side chain, and the density of these side chains is thought to provide some resistance of the protein core to protease digestion (Horner 1971).

Heparin has many known biological functions, most of which are mediated by binding of the heparin molecule to a protein. Antithrombin III is activated by interaction with heparin, which mediates its anticoagulant activity by promoting inhibition of several serine proteases involved with coagulation, namely thrombin (Rosenberg 1977), low molecular weight fractions of factor XII (Stead et al 1976), factors Xa, IXa (Oterud et al 1976), and XIa (Damus et al 1973). Inhibition of kallikrein (Burrowes et al 1975) and plasmin (Highsmith and Rosenberg 1974) is also potentiated. This anticoagulant activity is neutralized by other proteins with high affinities for heparin, such as protamine (Chargaff and Olson 1937) eosinophil major basic protein (Gleich 1977) and platelet factor 4 (Broekman et al 1975). Heparin is also involved in many cellular and humoral activities including regulation of the electronegative potential of vascular wall (Jaques 1967), fat metabolism (Wollin and Jaques 1974), the ovarian cycle (Lindner et al 1977) and a wide range of enzyme activities (Dawes 1982).

Heparin is present in mammalian connective tissue where it forms the matrix of the mast cell secretory granule to which many of the preformed basic mediators such as histamine bind by ionic interaction (Uvnas et al 1970).

Heparan Sulphate.

Heparin and heparan sulphate are considered to be members of the same family which includes a spectrum of molecules of different

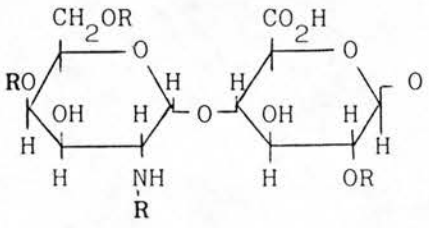
uronic acid composition and degree of sulphation, from highly sulphated, iduronic acid - rich polysaccharides (heparin) at one end of the spectrum and low-sulphated, glucuronic acid-rich species at the other (Lindahl and Hook 1978). Gallagher and Walker (1985) proposed that because of their differing patterns of sulphation, they should be classed as separate families of N-sulphated polysaccharides. They showed that more than 80% of glucosamine residues in heparin are N-sulphated and even more O-sulphated, whereas N-sulphate and N-acetyl groups were arranged in similar numbers and patterns along heparan sulphate molecules, while the O-sulphate groups varied considerably depending on the source of heparan sulphate. These molecular distinctions between heparan sulphate molecules have been further defined (Dietrich et al 1983) and a large number of different structural forms were found to exist, depending on the proportion of four basic disaccharide units, which vary according to tissue and species of origin.

The ability of heparan sulphate to inhibit the coagulation process is much less than that of heparin, and its major biological function is therefore less obvious. Heparan sulphate is abundant on many cell surfaces (Kraemer 1971a; Kraemer 1971b; Kjellen et al 1980) and it is thought to function in cell-cell recognition and adhesiveness (Dietrich et al 1977) due to its inherent molecular variability in different cell types (Dietrich et al 1983; Buonassisi and Colburn 1983).

Chondroitin Sulphate.

Chondroitin sulphates are GAGs with ß 1-4 linked disaccharide units of glucuronic acid in ß 1-3 linkage to galactosamine (Figure B).

Figure B.



R may be SO_3 or H.

The main subtypes of chondroitin sulphate are chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and a less abundant subtype chondroitin-4,6-disulphate (chondroitin sulphate E) where numbers designate the position of O-sulphation on galactosamine. Thus chondroitin sulphates have varying degrees of sulphation, and highly sulphated chondroitin sulphate can be mistaken for heparin.

All cells probably contain GAGs and each cell or tissue may contain one or more types; these also vary between species. For example rat peritoneal mast cells have been shown to synthesise not only heparin, but also chondroitin 4-sulphate, chondroitin 6-sulphate and a GAG containing a disulphated disaccharide, thought to be chondroitin sulphate E (Stevens and Austen 1982).

Methods for the measurement of heparin and related GAGs have to date been of two types based on

a. chemical properties and interaction with dyes,

or **b.** anticoagulant activity.

Chemical methods such as the uronic acid carbazole reaction (Bitter and Muir 1962), and metachromatic interactions with dyes such as Azure A (Jaques et al 1947) and toluidine blue (Jaques and Wollin 1967) can be applied to all GAGs, but do not distinguish between them. The assay of heparin by means of its ability to catalyse the hydrolysis of Auramine O (Band and Lukton 1982) is specific for

heparin, but is subject to interference from many components and will not detect concentrations of heparin below 1μ g/ml. Analysis of the monosaccharide constituents (Roden et al 1972; Blumenkrantz and Asbol-Hansen 1973) defines the type of GAG, but requires large amounts and cannot be used on a routine basis.

Methods based on the biological activity of heparin as an anticoagulant involve either direct measurement of heparin's inhibitory effect on in vitro blood coagulation (Marder 1970) or more specifically, quantitation of heparin enhancement of clotting enzyme inactivation by antithrombin III (Yin et al 1973) or factor Xa (Yin et al 1973; Teien et al 1976). These assays are subject to interference from clotting factors and inhibitors of coagulation (Yin et al 1973; Godal 1975) and due to problems stemming from the microheterogeneity of heparin preparations, there is considerable variability in the detection of different heparin preparations with different assay systems.

The ability of heparin-like polysaccharides to form insoluble complexes with serum lipoproteins (Srinivasan et al 1975) has been exploited by Wu and Cohen (1984) in the development of a competitive binding assay. It is based on the competition of naturally occurring GAGs (isolated from tissue digests) with radiolabelled heparan sulphate for the formation of precipitates in serum. The use of this method is however limited in that it will not detect concentrations of less than 10μ g/ml.

A competitive binding assay was developed by Dawes and Pepper (1982), based on the competition of unlabelled and radiolabelled GAGs for binding to a positively charged solid phase. The assay is sensitive to the degree of sulphation and molecular weight of the GAG, and will detect concentrations as low as 200ng/ml. This assay

was developed further and refined for application in the study of material contained in basophilic leucocytes.

(2). Materials and Methods.

Materials.

Reagents.

Tween 20, Triton X-100, trichloroacetic acid 'AnalaR' (TCA) and dimethyl sulphoxide 'AnalaR' (DMSO) were purchased from BDH Limited, Poole, Dorset. Opocrin (HTS/435), (heparan sulphate standard) was supplied by Dr R Mastacchi, Alfaricerche, Corlo, Italy. Organon 10172 was a kind gift of Dr H. Moelker, Organon, Oss, Netherlands. Porcine mucosal heparin (sodium salt, grade 1; Product No. H3125), bovine serum albumin (BSA),(Fraction V Powder; Product No. A4503), Pronase (bacterial protease Type 1V; Product No. P0384), chondroitinase ABC (Product No. C2905), lipoprotein lipase (Product No. L8634) and phospholipase C (Type V, Product No 4014) were all purchased from Sigma Chemical Company Ltd, Dorset, England. Heparan sulphate used for iodination was a gift from Dr E.A.Johnson (National Institution of Biological Standards and Control; London, UK). Polybrene [poly (N,N,N',N'-tetramethyl-N-trimethylenehexamethylene) diammonium bromide] was purchased from Aldrich Chemical Company Limited, Dorset, England. Sepharose 4B, Sephadex G-50 Superfine, Sephadex G-25 and Epoxy-Sepharose were purchased from Pharmacia (G.B. Ltd), Milton Keynes, England. ^{125}Iodine (^{125}I) for iodination was purchased from Amersham, Buckinghamshire, England.

Buffers.

Phosphate buffer.

To make a stock solution of 0.25M phosphate buffer, 100g of sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) was dissolved in 1 litre, and added to 16 litres of deionised water. 570g of di-sodium hydrogen orthophosphate ($NaHPO_4$), was dissolved in 2 litres of heated deionised water, and added slowly with mixing to previous 17 litres. This solution was then made up to 20 litres and adjusted to pH 7.4. This was then diluted 1:5 with deionised water for use at 0.05M.

Tween/phosphate buffer.

Phosphate buffer (0.05M) was made as required from the stock solution of 0.25M stored at room temperature. Tween 20 was added at 1% (weight/volume), and stored at 4°C for up to one week.

BSA buffer.

Bovine serum albumin (BSA) was added to Tween/phosphate buffer at a concentration of 10mg/ml. This buffer was susceptible to bacterial contamination, and was made fresh when required.

Sepharose 4B.

Sepharose 4B was filtered on a sintered glass funnel and washed thoroughly with phosphate buffer (0.05M, pH 7.4). The solid phase was then resuspended to original volume using phosphate buffer, and used at a dilution of 1:150 vol/vol to determine non-specific binding in the assay.

Polybrene-Sepharose 4B.

Polybrene was used as the binding reagent in place of the protamine described in the original method of Dawes and Pepper (1982), since it was more stable and not susceptible to protease digestion. This reagent was prepared by Dr DS Pepper. Polybrene was mixed with epoxy-Sepharose at pH 12 for 16h at 37°C. The loading was optimised at 12μ g Polybrene/ml of slurry. The product was thoroughly washed with 0.15M NaCl solution at pH 7.0, and was stable for approximately six months.

Methods.

Radioiodination.

Heparan sulphate 1 (NIBSC), the heparan sulphate Org 10172 and commercial sodium heparin (Sigma) were conjugated with 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (SHPP) by reacting the heparan sulphate or heparin (10mg) with an excess (10mg) of non-iodinated SHPP in 5ml of 0.05M sodium borate (pH 9.2) at 4°C for 20h. Unreacted ester, at this stage hydrolysed to the acid, was removed by gel filtration in water on Sephadex G-25. The material eluted at the void volume was chromatographed on a column of Polybrene-agarose (MacGregor et al 1984) to isolate the biologically active derivatives. These were desalted, lyophilised and iodinated with ^{125}I by the method of Greenwood et al (1963) as follows.

The reaction tube contained

10μ l of $Na^{125}I$ (1mCi)

10μ l of derivatised heparan sulphate or heparin (containing at least 500μ g/ml)

10µ l of Chloramine T (at 5mg/ml dissolved in 0.25M phosphate buffer immediately before use).

The mixture was allowed to react for approximately 10 seconds and then stopped by adding 0.86ml of $Na_2S_2O_5$ (160µ g/ml in 0.05M phosphate), followed by 100µ l of KI carrier (10mg/ml in 0.05M phosphate buffer).

The reaction tube was immediately counted on a NE Scaler-Ratemeter(SR3) γ -counter, and the contents transferred to a 1x10cm Sephadex G-50 column. The reaction tube was rinsed twice and finally counted. The column was run in Tween/phosphate buffer, and 20x1ml fractions were collected and counted. The stock tracer was stored in liquid nitrogen at -170°C in 20µ l aliquots, and was stable for approximately 3-4 months. Aliquots were thawed as required for assay, and diluted with Tween/phosphate buffer to a concentration of 10ng/ml.

Assay of Glycosaminoglycans.

The assay mixture contained

50µ l of biological sample or standard.

50µ l of Polybrene-Sepharose 4B.

50µ l of ^{125}I heparan sulphate I or heparin.

150µ l of BSA buffer or DMSO.

The concentration of Polybrene-Sepharose 4B used was predetermined by testing serial dilutions in the assay. The concentration chosen for use bound 50-60% of tracer in the absence of unlabelled material, and gave reproducible results with biological samples. The starting concentration for the heparin standard was 1µ g/ml, and eight doubling dilutions of this formed the standard curve. The starting concentration for heparan sulphate assay was 20µ g/ml, and

eight doubling dilutions were made. All standards and samples were assayed in duplicate. For each assay, standard curves were included both before and after all samples, to detect drift in the assay.

Maximum binding of the tracer was determined for each assay by including quadruplicate zero standard tubes, which contained 50µ l of Tween/phosphate buffer instead of 50µ l of standard or sample.

Non-specific binding of the tracer was determined for each assay by including duplicate samples where 50µ l of Tween/phosphate buffer replaced sample or standard, and 50µ l of Sepharose 4B replaced Polybrene-Sepharose 4B.

Four counting standard tubes containing only 50µ l of tracer were used to determine the time required to detect 10,000 counts.

All tubes were shaken at room temperature for 3h or 18h, and Sepharose-bound material was separated from that remaining in the liquid phase, by the method described by Hunter (1977). This involved sedimentation of the assay mixture through a solution of Tween/phosphate buffer containing 10% sucrose, which was dispensed using a multi-channel pump dispenser and manifold. Supernatants were removed after 20 minutes by aspiration. Tracer bound to Sepharose was then counted on a NE 1600 γ -counter (Nuclear Enterprises, Sighthill, Scotland), for the time determined from the counting standards.

Collection of Biological Samples.

Serum.

Whole blood samples were collected in 10ml volumes, and allowed to clot at room temperature. These were then centrifuged (110g; 15min; 4°C) and supernatant serum retained.

Plasma.

Whole blood samples were collected into a variety of anticoagulants at a dilution of 1:9 (vol anticoagulant/vol blood). These were ETP-[EDTA (78mM), theophylline (10mM) and prostaglandin E_1 (0.33μ g/ml), pH 7.4] , EDTA (50mM), or citrate (3.12% trisodium citrate in 5% HEPES buffer, w/v). The samples were then centrifuged (110g; 15mins; 4°C) and supernatant plasma retained.

Preparation of Biological Samples.

Biological samples were prepared for assay by incubating with Pronase (a non-specific protease isolated from Streptomyces griseus), at a final enzyme concentration of 5mg/ml at 37°C for 18-24h. The treated samples were then frozen to -20°C before assay.

Determination of Total Cell Heparan Sulphate.

Triton X-100.

A stock solution of 1% Triton X-100 (w/v) was made using 0.05M phosphate buffer. Whole blood samples were diluted 1:10 with this stock solution and treated with Pronase before assay.

Freeze/thaw x 6.

Whole blood samples were frozen to -20°C and thawed six times to ensure complete cell lysis. Samples were then centrifuged (800g; 4°C; 30min) to obtain supernatant and a cell pellet containing cell membranes and debris. Supernatants were removed and cell pellets resuspended to the original sample volume using Tween/phosphate buffer. Both supernatants and resuspended pellet solutions were treated with Pronase before assay.

Enzyme Digestion.

Chondroitinase ABC.

Chondroitinase ABC (5 units) was reconstituted to 1ml using 0.05M phosphate buffer. This was stored at -20°C in 100µ l aliquots. Chondroitinase ABC was added to samples at a final concentration of 0.2 units/ml. A control sample of chondroitin 4-sulphate in plasma (100µ g/ml) was used to test the activity of chondroitinase ABC in this system.

Lipoprotein lipase.

Lipoprotein lipase (100 units) was reconstituted to 2ml using 0.05M phosphate buffer to give a stock solution of 50 units/ml. This was added to samples at a final concentration of 1 unit/ml, and incubated for 24h at 37°C.

Phospholipase C

Phospholipase C was supplied as a stock solution containing 250 units/160µ l. This was diluted in 0.05M phosphate buffer, and added to samples at a final concentration of 1 unit/ml. Samples were then incubated at 37°C for 24h.

(3). Results.

Calculation of % yield and specific activity of radioiodinated tracer.

Specific activity and concentration of tracer were determined by the following method, (based on Greenwood et al 1963) illustrated with an example in Table 1. The elution profile of this radioiodination is shown in Figure 1. There were two peaks of radioactivity, a first peak containing iodinated GAG (Peak I), and a second peak (Peak II), containing unbound ^{125}I.

Table 1: **Calculation of the Percentage Yield and Specific Activity of Tracer following Chloramine T Radioiodination.**

Fraction No.	Counts/10 Seconds.
1	3
2	6
3	3
4	11624
5	43773
6	16546
7	5576
8	1958
9	1335
10	6751
11	11693
12	5302
13	1800
14	817
15	344
16	162

Total radioactivity added = 107639c/10sec (1000 μ Ci or 37000 kBq).

Total GAG added = 68μ g of derivatised heparan sulphate I

Total free ^{125}I eluted from column = 26896 counts.

Total counts associated with antigen = Total counts added-total eluted.

= 107693 - 26896

= 80824 counts.

% Yield = $\frac{80824}{107693} \times 100 = \underline{75.05\%}$

Specific activity = $\frac{0.7505 \times 1000}{68} = \underline{11.04\ \mu\ Ci/\mu\ g}$

Of 80824 counts associated with antigen, 71943 are in fractions 4,5 and 6. Thus fractions 4,5 and 6 contained $\frac{71943}{80824} \times 68 = 60.53$ ug/3ml

Thus stock tracer contained = $\underline{20.18\ \mu\ g/ml.}$

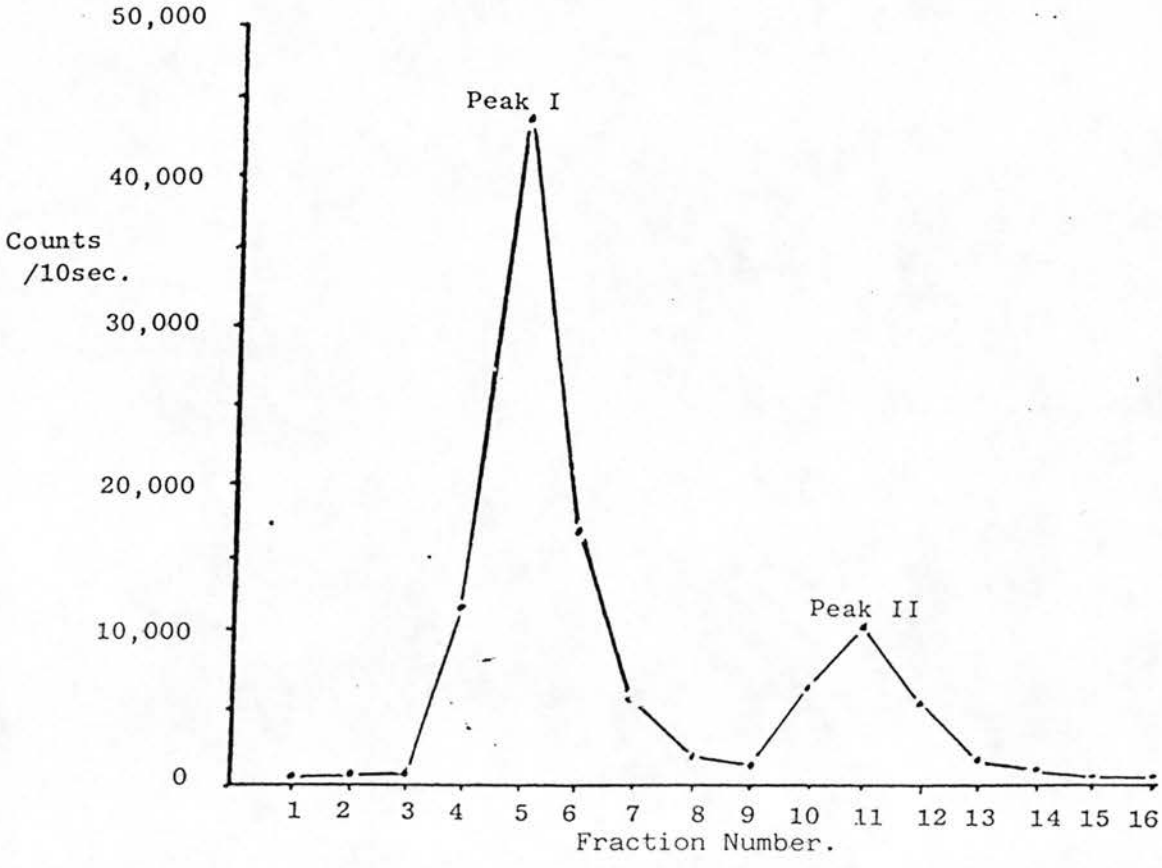


Figure 1: **Elution Profile of Radioiodination.**

Elution profile of iodinated GAG following reaction of ^{125}I with derivatised GAG, when purified on a Sephadex G-50 column. Two peaks of radioactivity were observed: ^{125}I-GAG (Peak I), and free ^{125}I (Peak II).

a. The total ^{125}I used for radioiodination was determined by counting the reaction tube before fractionation on the Sephadex G-50 column.

b. The radioactivity associated with GAG was calculated as being that in **a**, minus the counts eluted as free ^{125}I (Table 1) from the Sephadex G-50 column. This figure was then used to calculate the % yield of ^{125}I-labelled material, and its specific activity.

c. The counts in each fraction containing ^{125}I-labelled material were then expressed as a percentage of **b**, and used to calculate the concentration of radiolabelled GAG. This method assumes that all radioactivity that is not eluted as free ^{125}I is antigen associated, and may result in an overestimation. However, the error is likely to be small, as free ^{125}I (as iodide) does not readily adsorb to the reaction tube or the Sephadex G-50 column.

Measurement of standard and exogenous heparin.

The original assay used heparin as the standard and ^{125}I-labelled heparin as the tracer, in a buffer of 1% Tween/phosphate. A typical standard curve is shown in Figure 2. Various conditions were sequentially altered to optimise this system for use with biological samples. A variety of substances were tested in the standard assay to replace the 150µ l of 1% Tween/phosphate buffer in an attempt to improve reproducibility and stability of samples in the assay. These were

a. **Urea**: This was tested at 5M and 1M concentrations. A standard curve was obtained for both, but there was a considerable reduction in total binding.

b. **NaOH**: Binding curves were obtained, and appeared to be parallel

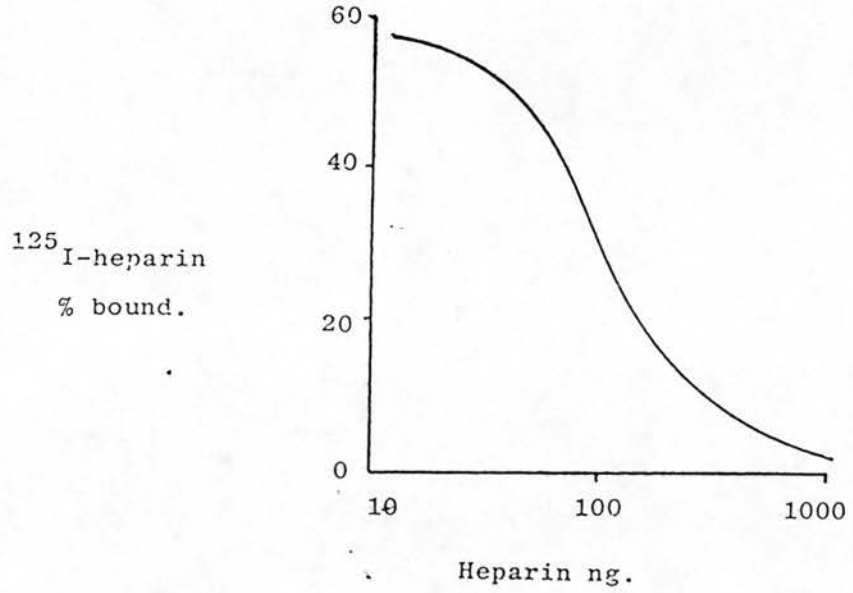


Figure 2: Heparin Assay Standard Curve.

with the standard when 0.5M NaOH or 1M NaOH and 1M NaCl were used. However, non-specific binding was increased to 35-40%, when it should have been less than 5%.

c. DMSO: Binding curves comparable to the standard were obtained when DMSO was included in the assay at 50% by volume.

d. BSA buffer: Binding curves obtained when BSA buffer was used at 10mg/ml gave low non-specific binding levels, but overall maximum binding was reduced, producing a curve with a less steep gradient than the standard.

BSA buffer and DMSO were tested using serial dilutions of fresh plasma spiked with heparin at 10mg/ml. They were equally suitable, and no interference from plasma constituents was detected in either system.

Four samples of plasma obtained from patients receiving heparin for therapeutic purposes were tested in this assay. All samples had high detectable levels of heparin, and assay of serial dilutions of each sample produced a curve parallel to that of standard heparin. This indicated that the material was similar to heparin, and was therefore directly comparable to the standard (Figure 3).

Measurement of endogenous material.

Human Serum.

Serum samples were obtained from 30 normal healthy controls, Pronase treated and assayed. Levels obtained ranged from 860-3850ng/ml (mean value = 1895.6ng/ml; SD=713.9ng/ml; SEM=130.3ng/ml).

Human Plasma.

Plasma samples were obtained from 3 individuals, using 3 different

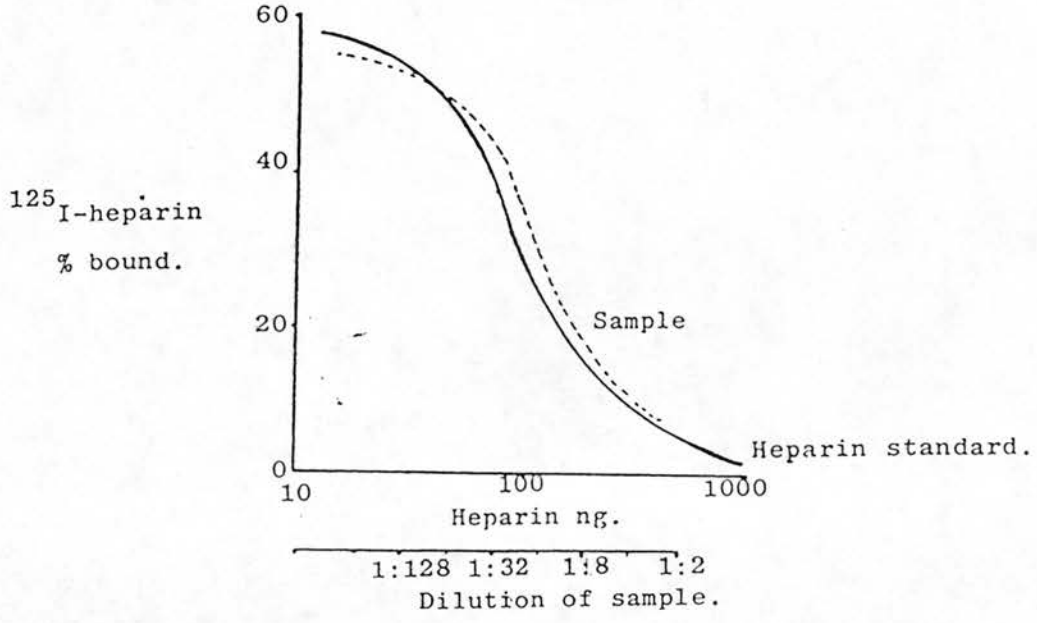


Figure 3: Assay of Exogenous Heparin.

Serial dilutions of plasma from a patient receiving heparin for therapeutic purposes were assayed, and compared with the standard heparin curve. Parallel curves were obtained.

anticoagulants (Table 2). Spontaneous release of material into plasma during sampling and handling of samples was minimal using citrate anticoagulant. Citrate anticoagulant was therefore chosen as the most appropriate anticoagulant to obtain plasma samples in this study. Plasma samples from 30 normal healthy controls were Pronase treated and assayed. Levels obtained ranged from 200 to 873 ng/ml (mean value = 443.8 ng/ml; SD=195.3ng/ml; SEM=35.6ng/ml).

Assay of endogenous material.

Plasma samples with and without Pronase treatment were serially diluted and assayed, using both BSA buffer and DMSO heparin assay systems. Detectable levels were obtained from Pronase treated samples only, but when serially diluted, did not produce a curve parallel to the heparin standard (Figure 4). Various combinations of standard and tracer were then screened for an appropriate system for assay of the naturally occurring material in plasma.

Heparan sulphate I tracer was used in place of heparin tracer, and Opocrin (heparan sulphate I) replaced heparin as the standard (Figures 5a and 5b).

DMSO caused interference and reduced binding considerably in the heparan sulphate assay. The curve was very flat and could not be used as a standard curve (Figure 5b).

The BSA buffer system detected the heparan sulphate standard, and produced a satisfactory standard curve which was less steep than that of the heparin standard. This system also detected material in Pronase treated biological samples, with reproducible results (Figure 5a).

Table 2: **Collection of Plasma Samples using Different Anticoagulants.**

	Control 1	Control 2	Control 3
ETP	900	860	732
EDTA	676	585	738
Trisodium citrate	599	554	599
Serum	2815	1915	2288

Samples were collected from three healthy controls, and assayed for GAG. Results are expressed as ng/ml.

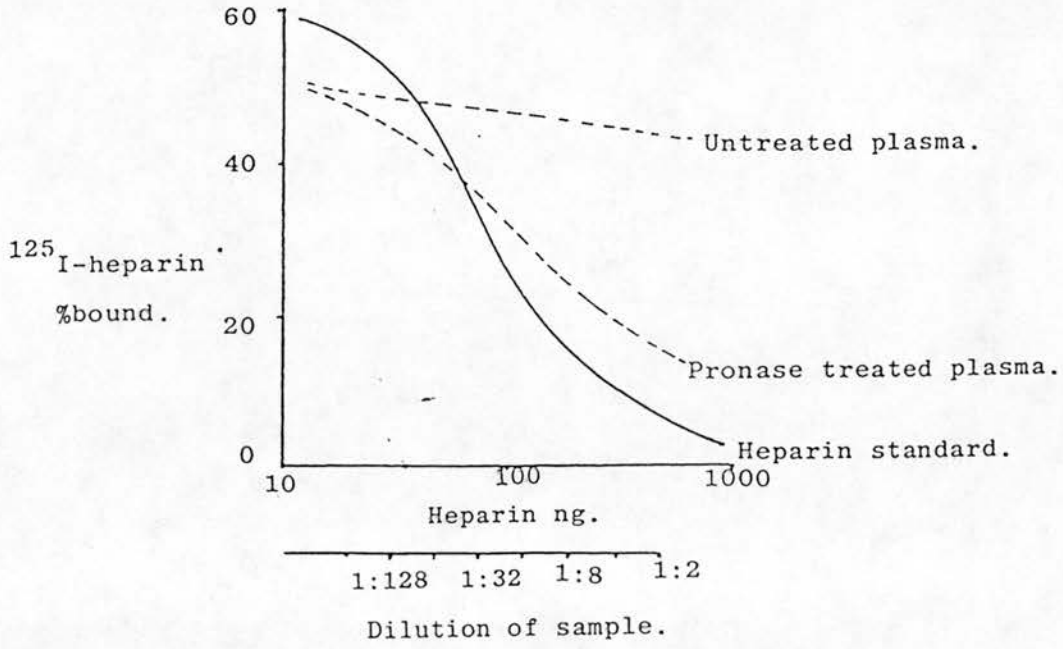


Figure 4: **Measurement of Endogenous Material in Heparin Assay.**

Fresh human plasma samples with and without Pronase digestion were serially diluted, assayed and compared with the standard heparin curve. Material was detected in Pronase treated plasma only, and its dilution curve was more horizontal than that of standard heparin.

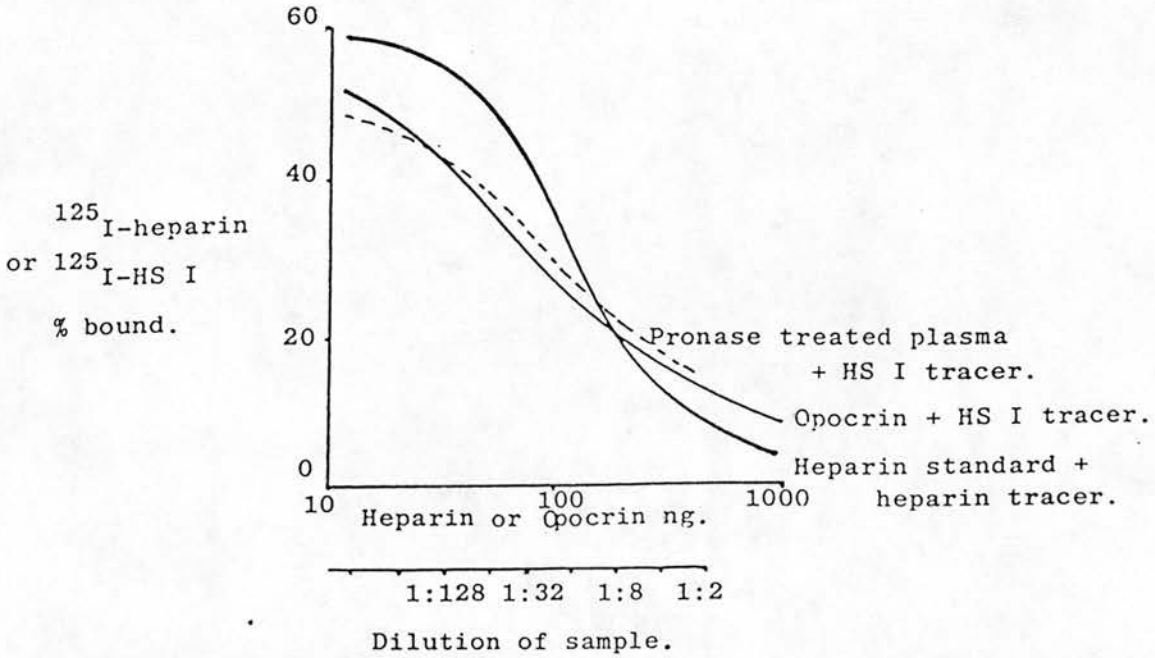


Figure 5a.

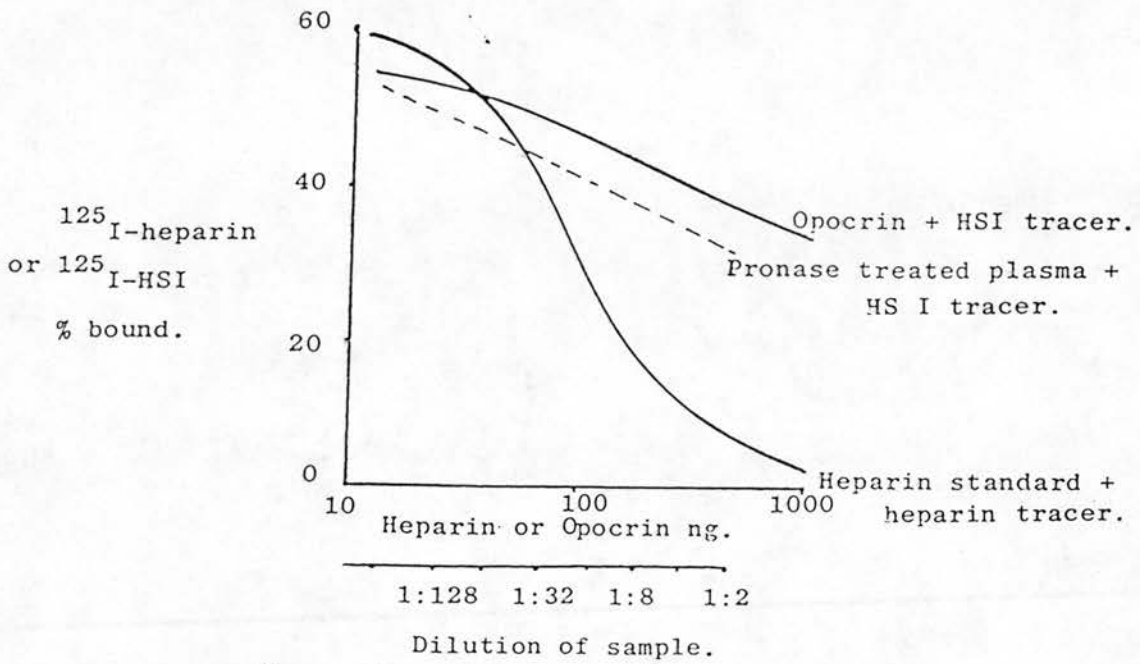


Figure 5b.

Figure 5: **Assay of Endogenous Material in Heparan Sulphate Assay.**

The assay of heparan sulphate standard, using heparan sulphate I tracer, was compared with the standard heparin assay in the presence of **a.** BSA buffer, and **b.** 50% DMSO. Serial dilutions of Pronase treated plasma were also included, to test the activity of naturally occurring endogenous GAGs with heparan sulphate I tracer. Heparan sulphate-like material was detected in the presence of BSA buffer, and DMSO clearly interfered with its assay.

Organon 10172 (Org 10172) was tested as the standard, and iodinated Org 10172 was used as tracer. The graphs produced were parallel to those using heparan sulphate I and Opocrin. A correlation curve of the Opocrin/Org 10172 standards was drawn (Figure 6), and revealed limited scattering of points, especially on the middle "working area" of the curves. There was less correlation at lower concentrations, due to differing degrees of sulphation of the two materials. Having established the most appropriate combination, that of heparan sulphate I tracer and Opocrin standard, a range of specific GAG standards were tested (as dilution curves) to determine those detectable with this system (Figure 7). Heparan sulphate I, dermatan sulphate and chondroitin sulphate E were all measurable at (approximate concentration range) 1-10μ g/ml, and produced dilution curves parallel to the Opocrin standard. Heparin was also detected, but was distinguished from the other GAGs tested by its characteristic steep curve. Chondroitin 4-sulphate, and chondroitin 6-sulphate were also detected, but only when present at concentrations of greater than 1mg/ml.

Effect of freezing on Pronase treated samples.

Doubling dilutions of biological samples treated with Pronase gave a curve parallel to the heparan sulphate I standard curve, although reproducibility was poor at lower concentrations, reducing the sensitivity of the assay. Pretreated samples were frozen to -20°C before assay, which eliminated this problem.(Figure 8)

Freezing samples after digestion with Pronase may remove interfering components (possibly by precipitation), and therefore background disturbance. The intra-assay and inter-assay CV's for frozen/thawed, pronase treated samples was 10.7% and 4.6%

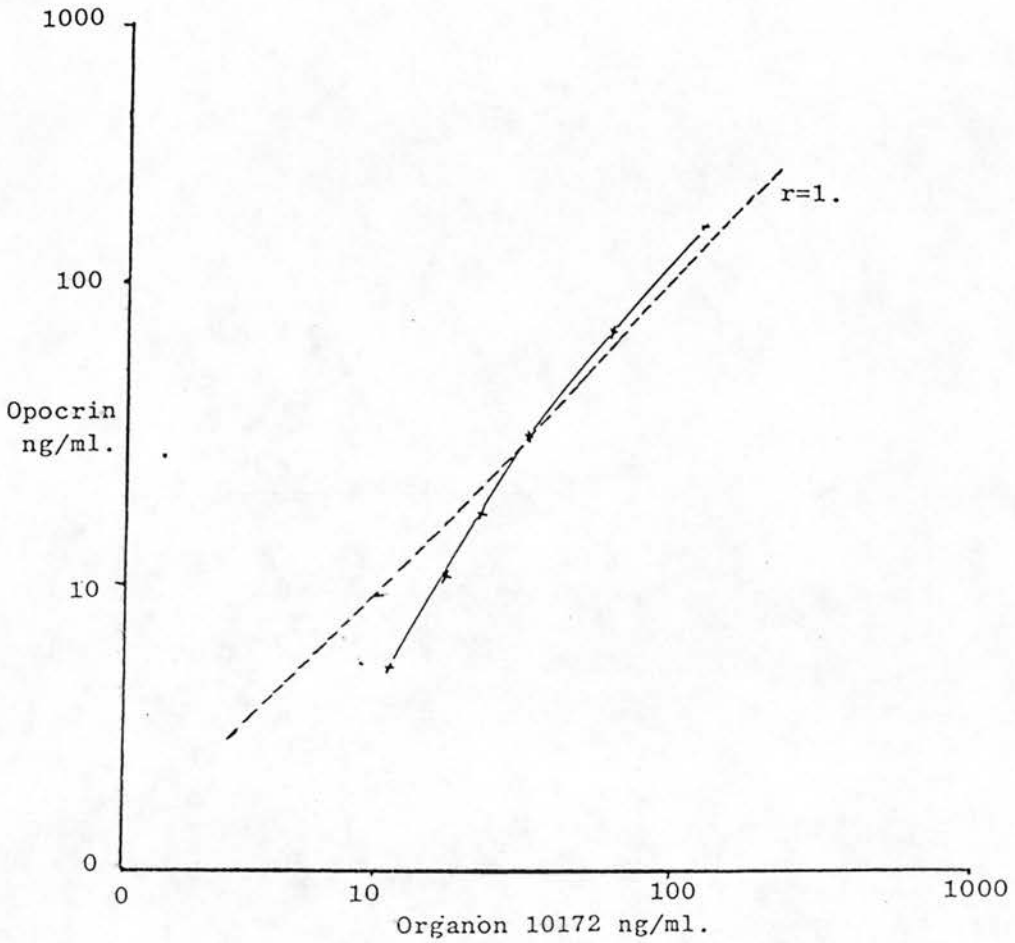


Figure 6: Correlation Curve of Opocrin and Organon 10172 Standards.

Both heparan sulphate standards, Opocrin and Organon 10172 were assayed over the same concentration range, using heparan sulphate I tracer and BSA buffer in the assay. The graph shows there was good correlation between the two standards, which was slightly reduced at the lower concentrations.

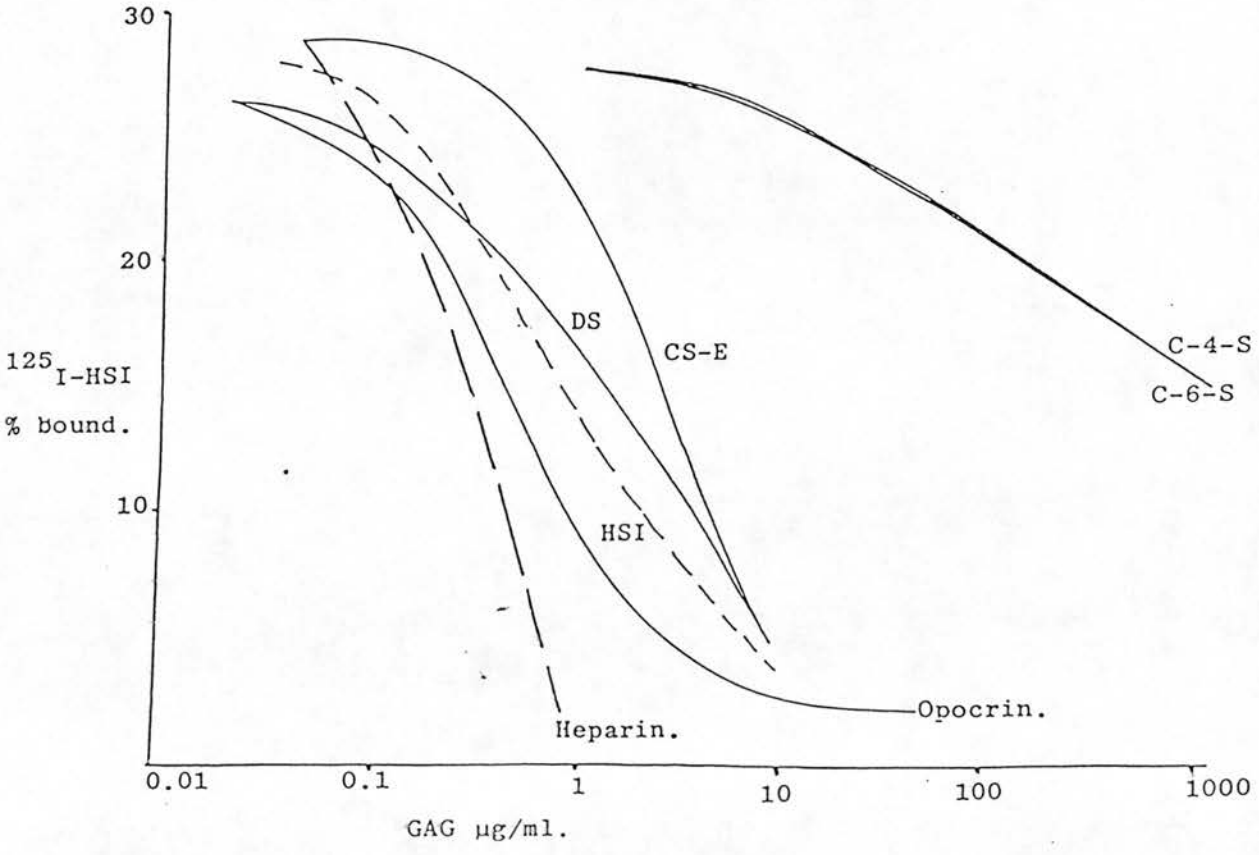


Figure 7: **Glycosaminoglycans in Heparan Sulphate Assay.**

Serial dilutions of a range of different GAGs were assayed using heparan sulphate I tracer, and BSA buffer. These were Opocrin, standard heparin, heparan sulphate I (HSI), dermatan sulphate (DS), chondroitin sulphate E (CS-E), chondroitin 4-sulphate (C-4-S), and chondroitin 6-sulphate (C-6-S).

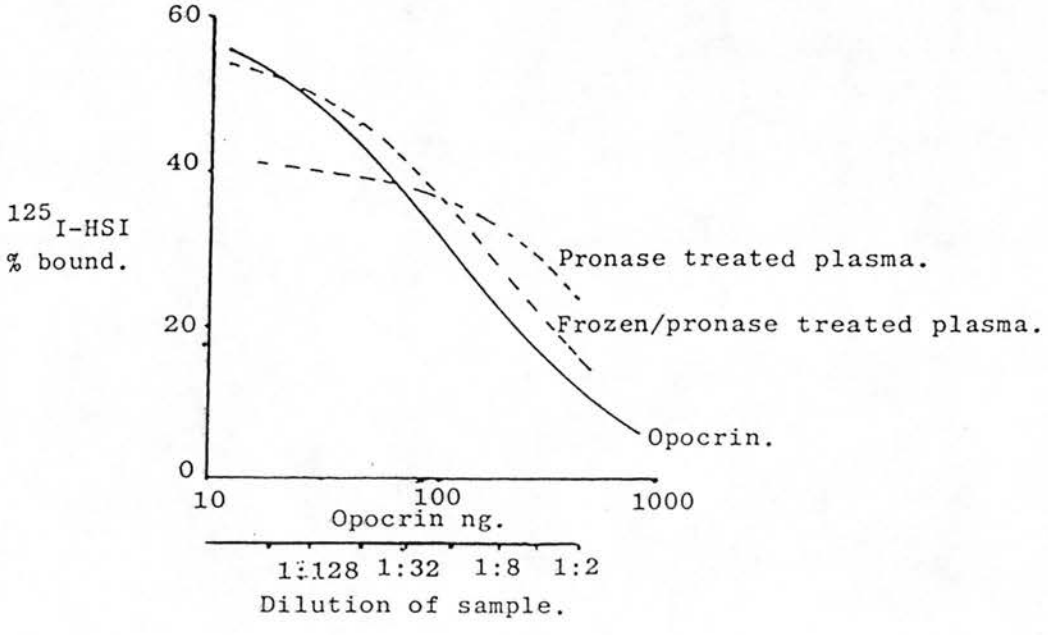


Figure 8: **Effect of Freezing on Pronase Treated Samples.**

Serial dilutions of Pronase treated plasma, before and after freezing and thawing once were assayed, and compared with the standard Opocrin. Both dilution curves were parallel with standard Opocrin; the frozen/thaw sample had greater reproducibility and stability at lower concentrations.

respectively. However, repeated freeze/thaw of samples tended to decrease their stability in the assay, and reproducibility became poor. Samples frozen and thawed more than twice were discarded.

Variation of shaking time.

Two standard curves and nine pretreated plasma samples were prepared for assay in duplicate. One set was shaken at room temperature for 3h, and the other for 18h before separation of the solid phase.

Standard curves were similar for each assay, but results obtained from plasma samples were very different (Table 3).

Biological samples were therefore not stable at room temperature overnight. Three hours of shaking appeared to be sufficient to allow the assay mixture to equilibrate, but not to allow incubation interference.

Determination of total cellular material.

To measure levels of total GAG contained in whole blood samples, cells were initially lysed by the following method.

Triton X-100.

Triton X-100 was added to whole blood samples at a final concentration of 1% to cause complete cell lysis by dissolving the membrane, and thus allow detection of total GAG contained in all blood cellular fractions. A high concentration of material was detected in whole blood treated in this way, but doubling dilutions of these samples did not produce a curve parallel to doubling dilutions of Pronase treated plasma, or heparan sulphate in the assay (Figure 9). The heparan sulphate standard was set up in

Table 3: **Variation of Shaking Time in Assay.**

Sample No.	Shaking Time 3h.	Shaking Time 18h.
1.	440	3560
2.	560	3480
3.	515	2680
4.	361	2450
5.	449	2510
6.	553	1880
7.	610	2730
8.	535	3050
9.	615	3460

Results are expressed as ng/ml.

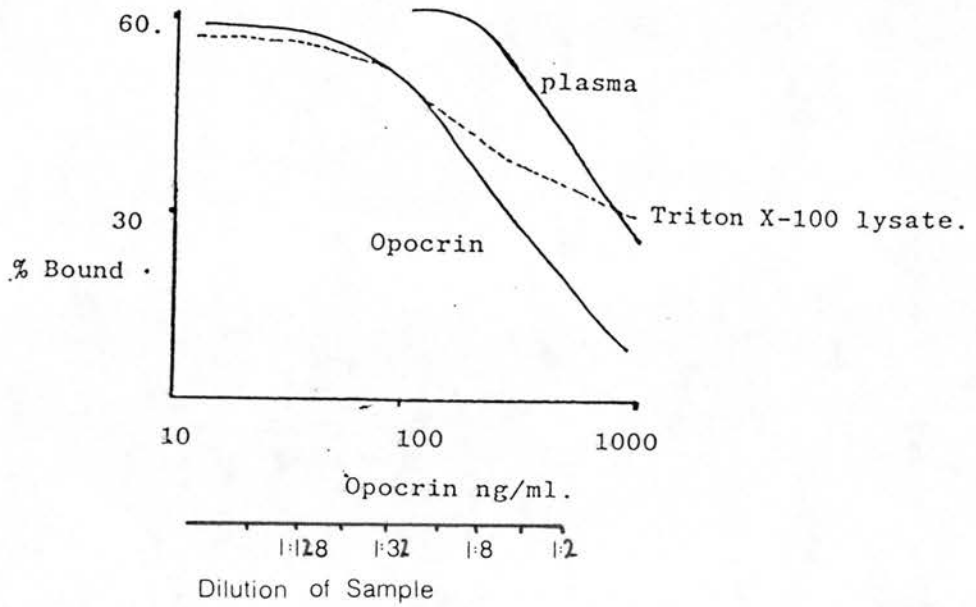


Figure 9: **Assay of Pronase Treated Plasma and Triton X-100 lysate.**

Serial dilutions of Triton X-100 lysates were assayed and compared with those of frozen/thawed, Pronase treated plasma, and standard Opocrin. The dilution curve of Triton X-100 lysate sample was more horizontal than those of the plasma and standard Opocrin curves, which were parallel to each other.

duplicate: one diluted with Triton X-100, and the other set up as normal. Both curves were identical when assayed, which ruled out interference from Triton X-100.

The lack of parallelism of the Triton X-100 lysate indicates that the material being detected in the assay was not directly comparable to the standard used. Alternative methods were therefore tested to release total GAG contained in whole blood samples. Methods tested were as follows.

Freeze/thaw x 6.

Cells were lysed by freezing (to -20°C) and thawing (to room temperature) untreated whole blood samples six times. Since membrane material was not dissolved by this method, samples were centrifuged to remove membranes and cellular debris.

Serial dilutions of Pronase treated supernatants and resuspended membrane pellets were assayed, and it was found that interfering components were contained in the membrane fraction. Material released into the supernatant fraction produced a dilution curve parallel, and was therefore directly comparable with those of both plasma and heparan sulphate I standard. However the absolute values of GAG contained in these supernatant samples were all < 1000ng/ml, much less than was released into serum. Thus this method did not successfully measure total content of GAG in whole blood.

Since the material detected from the resuspended cell pellet produced the same pattern as that of the Triton X-100 lysate (Figure 10), it was assumed that the interfering components were confined to the membrane fraction. To determine the nature of the material or interfering component of the membrane fraction, the Triton X-100 lysate was treated with a variety of enzymes before Pronase treatment as follows.

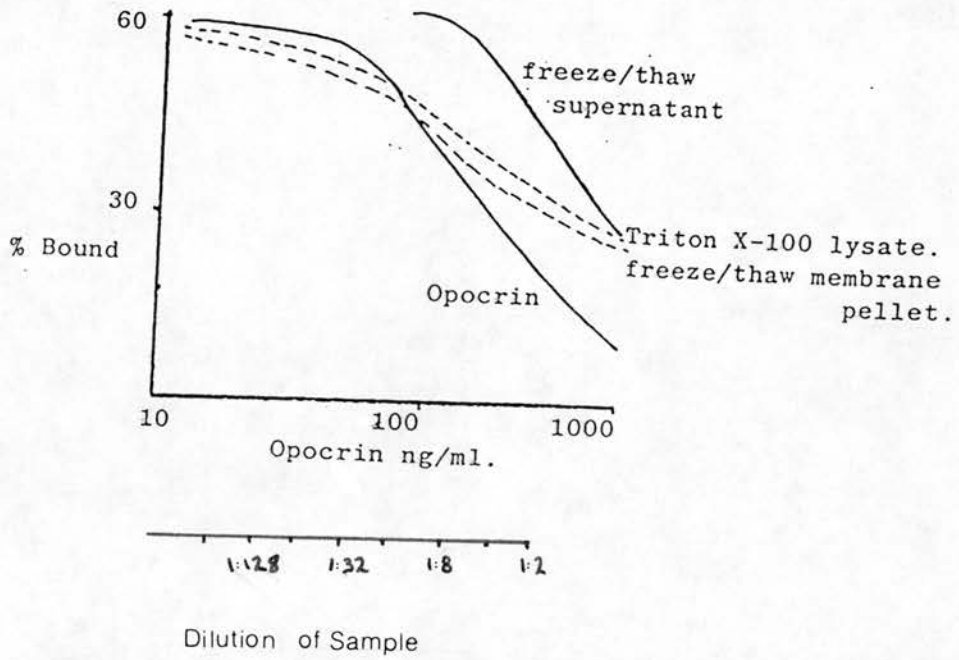


Figure 10: **Assay of Freeze/Thaw Supernatant and Resuspended,**

Membrane-Containing Cell Pellets.

A sample of whole blood was frozen and thawed six times, centrifuged and serial dilutions of both supernatant and resuspended cell pellet were assayed. These were compared with serial dilutions of Triton X-100 lysates and standard Opocrin. Supernatant sample dilution curve was parallel, and directly comparable with standard Opocrin, whereas the resuspended cell pellet produced a dilution curve with a more horizontal slope, parallel with the Triton X-100 lysate sample.

a. Chondroitinase ABC

To test if the material being detected in the membrane fraction was chondroitin sulphate, the effect of chondroitinase ABC on a sample of Triton X-100 lysate was tested. Control samples of lysate contained commercial chondroitin 4-sulphate at a final concentration of 100μ g/ml. Overnight incubation with chondroitinase ABC degraded the chondroitin 4-sulphate in the control sample, and at the lowest dilutions of Triton X-100 lysates, binding was reduced by 55.6% with chondroitinase ABC digestion, although detectable material was still present after this treatment (Figure 11). The interference detected in the lysate samples could therefore have been partly due to the presence of a chondroitin sulphate component in the membrane fraction.

b. Lipoprotein lipase.

Lipoprotein lipase treatment did not affect the response of the Triton X-100 lysate in the assay, since dilution curves of the lysate samples were similar before and after treatment (Figure 12).

c. Phospholipase C.

Digestion of a Triton X-100 lysate samples with phospholipase C yielded material which was readily assayed, and doubling dilutions of the treated lysate gave a curve parallel to the standard (Figure 13). Interference detected with the lysate (possibly by phospholipids in the membrane fraction) had been effectively removed by predigestion of the sample with phospholipase C.

Having established that the interference in the lysate samples was due to components confined to the membrane fraction, further methods for release were tested.

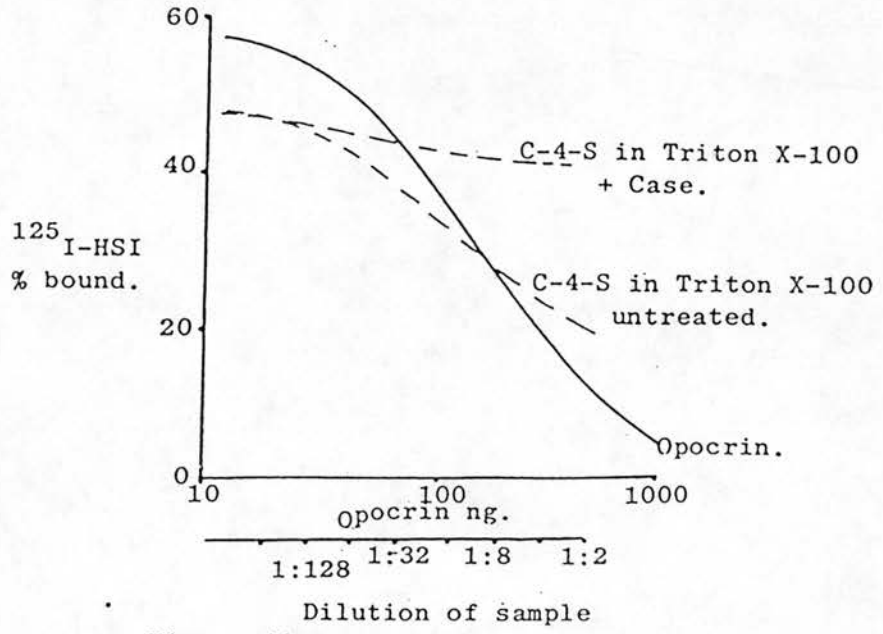


Figure 11a.

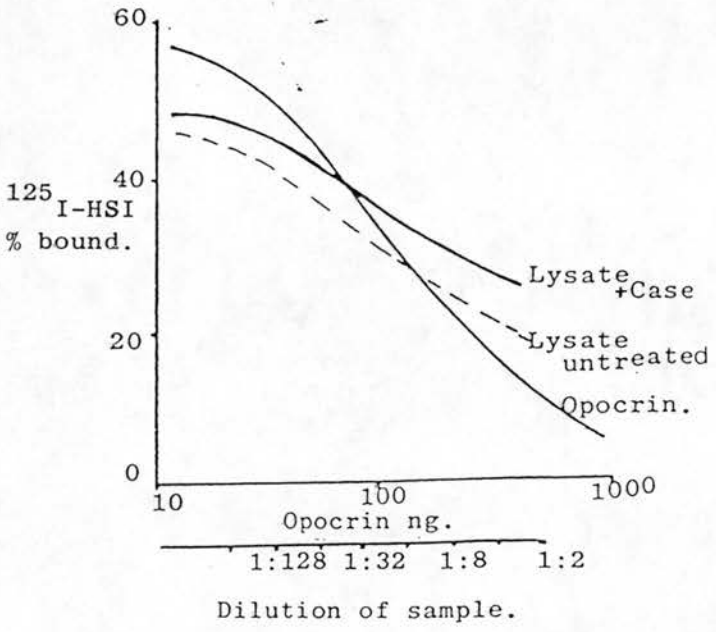


Figure 11b.

Figure 11: **Digestion with Chondroitinase ABC.**

Chondroitin 4-sulphate was diluted in Triton X-100, and treated with chondroitinase ABC. Serial dilutions of these chondroitinase digested and untreated samples were prepared in Triton X-100, and assayed. Chondroitin 4-sulphate was detected and produced a relatively horizontal dilution curve, which was degraded in the presence of chondroitinase ABC (Figure 11a). A Triton X-100 lysate sample was treated with chondroitinase in the same way, resulting in only partial degradation of material (Figure 11b).

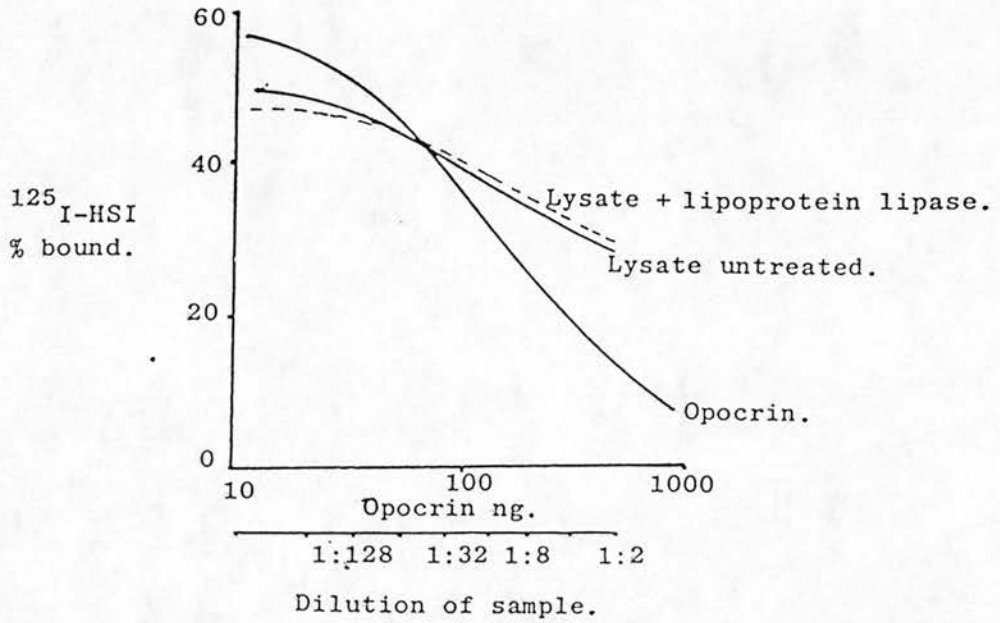


Figure 12: **Digestion with Lipoprotein Lipase.**

Serial dilutions of Triton X-100 lysate samples, with and without digestion with lipoprotein lipase, were assayed and compared with standard Opocrin. Incubation with lipoprotein lipase had no effect on the assay of Triton X-100 lysates.

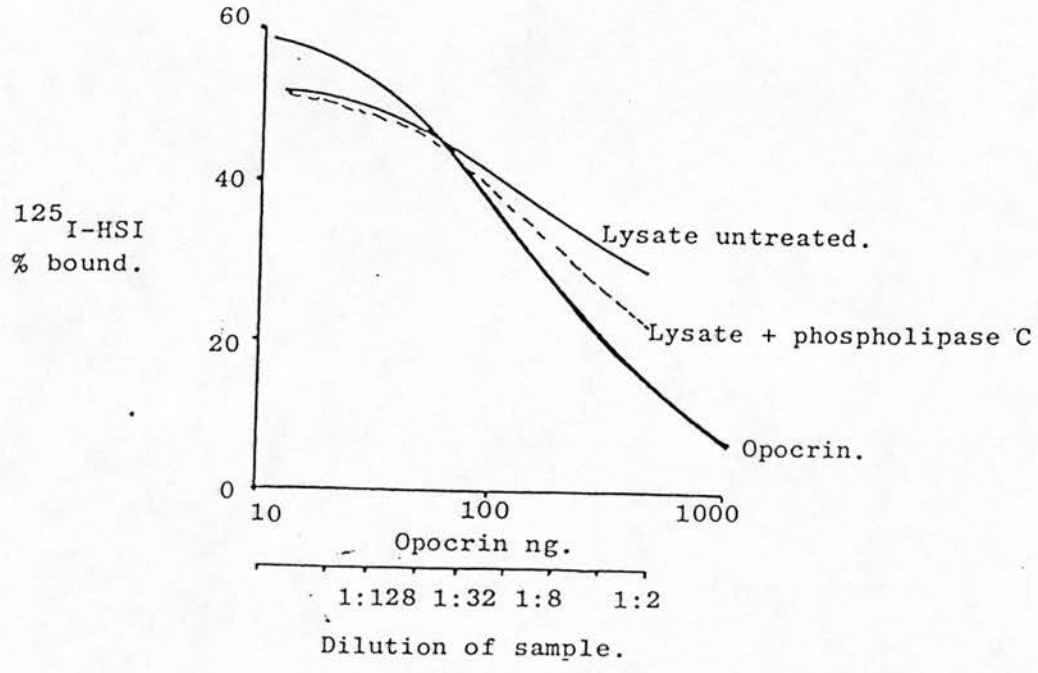


Figure 13: **Digestion with Phospholipase C.**

Serial dilutions of Triton X-100 lysate samples with and without digestion with phospholipase C, were assayed and compared with standard Opocrin. Increased levels of material were detected after digestion with phospholipase C, and the dilution curve became more parallel with that of standard Opocrin.

Tween 20.

A 2% Tween 20 solution was used as an alternative lysing agent to Triton X-100, as it was thought to dissolve only certain membrane components (possibly leaving those causing assay interference which could be removed by centrifugation). Centrifugation was necessary to remove undissolved membranes, which were retained and resuspended to the original sample volume with 0.05M phosphate buffer. Both supernatants and resuspended cell pellet samples were serially diluted and assayed as before (Figure 14).

The dilution curves of these samples showed that material released by cell lysis with Tween 20 was directly comparable with heparan sulphate standard. Material associated with interfering membrane components were removed from each sample by centrifugation. However, the absolute values of GAG detected in the supernatant samples were again < 1000ng/ml and clearly did not reflect the total content of GAG in these whole blood samples. Increasing the concentration of Tween 20 to 4% slightly increased the 'releasable' material detected (by approximately 2.5%). These levels of material detected were however still too low to be defined as total cellular content.

Release Without Membrane Solubilisation.

Methods to release total cellular material which did not involve the solubilisation of membranes were tested as follows.

a. Deionised water (Hypotonic lysis).

Whole blood samples were diluted 1:2-1:10 with deionised water, Pronase treated and assayed without centrifugation. No detectable material (< 200ng/ml) was present in any of the samples, and this method was therefore unsuitable to detect total cellular GAG.

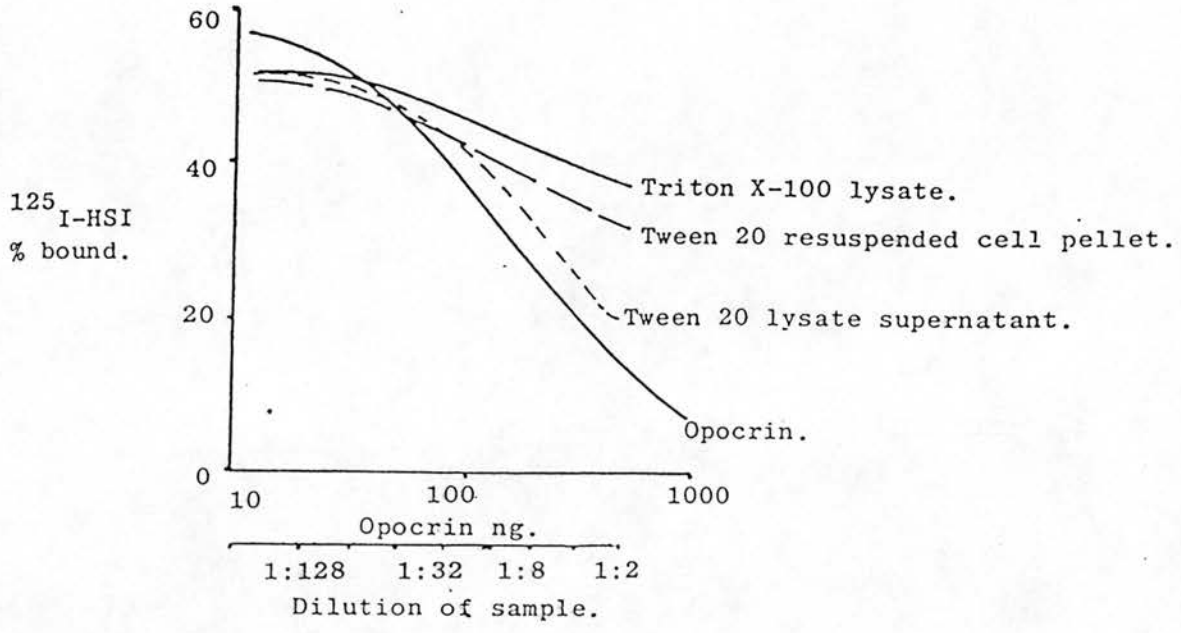


Figure 14: **Cell Lysis with Tween 20.**

Whole blood was diluted with 2% Tween 20, centrifuged, and serial dilutions of both supernatant and resuspended cell pellet were assayed. These were compared with serial dilutions of Triton X-100 lysate and standard Opocrin. The supernatant sample dilution curve was parallel and directly comparable with the standard Opocrin, whereas the resuspended cell pellet produced a dilution curve with a more horizontal slope, parallel with the Triton X-100 lysate sample.

b. Trichloroacetic acid (TCA).

This was added to samples at a final concentration of 0.5%, but interfered with the standard curve, and was therefore unsuitable.

c. Magnesium chloride ($MgCl_2$)

Free Ca^{++}, required for both the release mechanism and the clotting mechanism was chelated by diluting whole blood samples 1:10 with citrate anticoagulant, and $MgCl_2$ was added to at a final concentration of 0.2M. At this concentration, Mg^{++} is thought to mimic Ca^{++} ions in such a way that leucocyte release occurs, whereas the clotting mechanism remains inactive. These whole blood samples were centrifuged and supernatants Pronase treated and assayed as before. All samples produced levels of < 200ng/ml, and clearly GAG release from cells had not occurred.

d. Variation of citrate concentration.

Citrate was normally used at 3.12% (w/v) to chelate existing sources of Ca^{++} in whole blood samples, and inhibit the clotting mechanism. It was tested at half strength (1.56%) and also 1.25%, and 1% (w/v), in an attempt to prevent coagulation, but allow sufficient Ca^{++} to remain in solution, and allow release of GAG. Two whole blood samples were tested in this way, centrifuged, and supernatants were Pronase treated and assayed. (Table 4)

These levels were not much higher than would be expected of plasma samples (in full strength citrate levels of 200-873ng/ml can normally be expected), and release of total GAG had clearly not occurred. Citrate could not be tested at a concentration less than 1%, since clotting occurred in the samples. A combination of 1.56% citrate and 0.2M $MgCl_2$ added to the two whole blood samples, was also tested. Levels of 450 and 622 were obtained and again were levels similar to plasma, rather than total levels released from

Table 4: Variation of Concentration of Citrate Anticoagulant.

Concentration of Citrate Anticoagulant.	Control 1.	Control 2.
Full Strength (3.12%).	393	256
Half Strength (1.56%).	972	266
Citrate only at 1.25%.	690	392
Citrate only at 1%.	650	495
Citrate at 1.56% and 0.2M $MgCl_2$.	622	450

Samples were collected from two healthy controls, and results are expressed as ng/ml.

basophils contained in the samples.

e. Recalcification.

Whole blood was taken into full strength citrate as before, plasma was removed, and the cell fraction resuspended to its original volume with 0.1M calcium chloride ($CaCl_2$). These were incubated at 37°C for either 4h or 24h and then centrifuged. Supernatant samples were Pronase treated and assayed, giving levels of 200 and 240 ng/ml. Again it was clear that release had not occurred.

(4). Discussion.

The purpose of the studies described in this section was to develop further the assay of Dawes and Pepper (1982), and validate its use in the detection and characterization of GAGs associated with human basophilic leucocytes.

The assay was first improved by replacing protamine (used in the original assay system) with Polybrene. This, covalently linked to solid phase (Sepharose) bound the highly negatively charged heparin-related GAG molecules with high affinity. Moreover, this reagent, unlike protamine-Sepharose, was extremely stable which greatly increased its shelf life (up to 9 months in aqueous suspension at 4°C); in addition, it was not susceptible to digestion with Pronase, which obviated the need for inactivation of the Pronase used in sample pre-treatment. Standard heparin was iodinated as described, and an example of the curve obtained with the assay of standard heparin is shown in Figure 2. The stability and reproducibility of the assay was increased by including BSA buffer or DMSO (50% by volume) in all tubes. The addition of fresh plasma to standard heparin caused no interference in the assay, even with 24h incubation at 37°C before assay. Similar dilution

curves were obtained from plasma samples of patients receiving commercial heparin for therapeutic purposes (Figure 3), and predigestion with Pronase was not required for its detection. This could be regarded as 'in vivo' addition of heparin to plasma, and the detection of this added exogenous material was in no way altered by its incubation in plasma.

The assay of naturally occurring endogenous GAG was less straight forward. Firstly predigestion of samples with Pronase was essential, since naturally occurring GAGs were bound to a variety of proteins in vivo, and the assay was subject to interference from these proteins. It was not possible to measure material from untreated biological samples, but predigestion with Pronase yielded material which was readily assayed. However, the levels of GAG detected were low, and dilution curves, where measureable, were not parallel with that of standard heparin (Figure 4).

The most important factor influencing the binding affinity of a GAG to Polybrene is its degree of sulphation. The greater the degree of sulphation of a GAG, the greater its capacity to compete for binding sites. Since heparin is very highly sulphated, it had the greatest affinity for Polybrene of the GAGs tested. Less sulphated GAGs will compete less effectively with standard radiolabelled heparin, and will therefore appear to be present in lower concentrations than they actually are. It was therefore necessary to develop a system using a standard GAG and tracer as similar as possible to the GAG naturally occurring in biological samples. The appropriate assay system was achieved by replacing the heparin standard in the original assay with Opocrin and ^{125}I-heparin tracer with ^{125}I-heparan sulphate tracer. Dilution curves of Pronase treated plasma were parallel to the standard curve obtained using

this new system (Figure 5a), although in the presence of DMSO binding was severely reduced (Figure 5b). DMSO is thought to affect hydrophobic interactions. The structure of heparin is such that hydrophobic bonding is much less important than in less sulphated structures such as heparan sulphate molecules. The use of DMSO in the assay system will therefore discriminate between these GAGs and will not allow the detection of less sulphated GAGs (such as heparan sulphate), which have lower binding affinities to begin with. Using BSA buffer in place of DMSO, heparan sulphate-like material was detected in biological samples. Dilution curves of Pronase treated plasma were parallel with the standard curves of two available heparan sulphate standards, Opocrin and Org 10172. These two standards were compared, and correlation (Figure 6) was good at high concentrations of the standards, to well after the mid point (that is, the working area) of the curve. At lower concentrations however, the correlation was slightly reduced, probably due to slight differences in their degrees of sulphation. Thus the use of either standard should produce similar results at concentrations greater than 200ng/ml, and samples with levels below this were not read. Opocrin was chosen as the heparan sulphate standard for this study, and used consistently throughout.

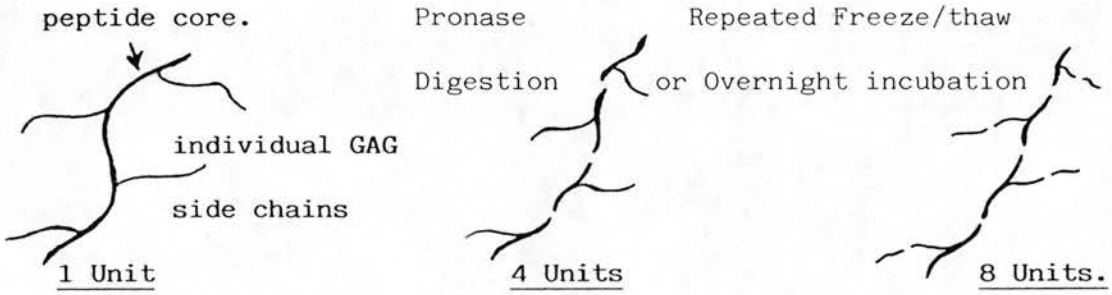
The properties of this assay system were such that larger, highly sulphated GAG molecules should be more readily assayed than smaller molecules of low charge density. The various GAGs tested in this assay behaved accordingly (Figure 7). The GAGs tested with a similar degree of sulphation were heparan sulphate I, Opocrin, dermatan sulphate, and an oversulphated chondroitin sulphate - chondroitin sulphate E. The dilution curves of these GAGs were more or less parallel to each other, and were measurable over a similar

concentration range (approximately 1-10μ g/ml). The highly sulphated heparin standard was assayed more readily than these, and its high affinity for Polybrene and effective competition with the heparan sulphate tracer produced a very steep dilution curve, measurable over a much lower concentration range (approximately 0.1-1μ g/ml). At the other end of the scale, chondroitin 4-sulphate and chondroitin 6-sulphate (differing only by the position of sulphate substitution on galactosamine) gave a dilution curve with a shallow slope, and were measurable only at concentrations of greater than 1mg/ml. On this basis therefore, the material detected in biological samples could be heparan sulphate, chondroitin sulphate E or dermatan sulphate. However, dermatan sulphate is mainly restricted to connective tissue and is thought not to circulate in peripheral blood in appreciable amounts; it therefore probably does not constitute the GAG measured in this study. Studies to further characterize the endogenous GAG detected are discussed in Chapter 3.

Doubling dilutions of human plasma, human serum and supernatants of freeze/thaw preparations (that is, lysed whole blood with the membrane fraction removed) all produced curves parallel to standard Opocrin. However, at the lower concentrations stability was reduced and reproducibility was poor. Factors contributing to this interference were removed by precipitation, since reassay of samples after freezing to -20°C increased the sensitivity, and was a useful step to ensure minimal background interference.

Repeat assays of aliquoted samples frozen/thawed only once produced reproducible results (r=0.98), but repeated freeze/thaw of individual samples increased the levels detected. Moreover, the levels increased more with each freeze/thaw. This was probably due

to the structural characteristics of the molecules assayed. GAGs are found in covalent association with proteins to form proteoglycans (Lindahl and Hook, 1978).



Pronase digestion releases the individual side chains, each representing 1 heparan sulphate equivalent in the assay. Repeated freeze/thaw of the Pronase digests may have caused further breakdown of the chains, resulting in many smaller GAG units, each with the capacity to bind to Polybrene, and thus increasing the apparent content of GAG in the sample. This view is supported by the finding that overnight incubation of the Pronase treated samples at room temperature produced the same effect as this freeze/thaw process (Table 3). Endoglycosidases contained in the sample may act during this long incubation period and cleave the carbohydrate backbone in the same way as the freeze/thaw procedure, increasing the apparent GAG content. Care was therefore needed to ensure that all biological samples were frozen/thawed as infrequently as possible, and the incubation period of the assay was minimal (3h).

GAGs are present in the connective tissue of mammals (Comper and Laurent 1978; Mathews 1975), on cell surfaces (Kraemer 1971a; Kraemer 1971b; Kjellen et al 1980) and in circulating basophilic leucocytes (Hakomori 1965; Olsson et al 1970). In an attempt to measure the total content of endogenous GAG in peripheral whole

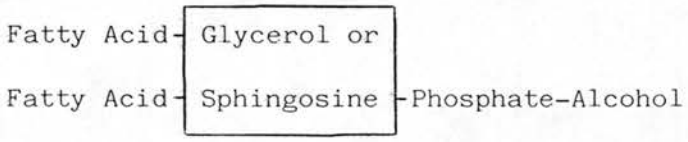
blood (that is, plasma content plus GAG within circulating blood cells), whole blood lysates were prepared and assayed. A solution containing the detergent Triton X-100 was used to dissolve membranes and release GAG molecules contained in blood cells. The material detected in these lysates was not directly comparable with the material measured in plasma, or with standard Opocrin (Figure 9). Since large amounts of protein as well as membrane fractions were present in these samples, it was not altogether surprising that some interference in this sensitive assay system occurred. Removal of the membrane fractions after cell lysis (with the freeze/thaw method) eliminated this interference (Figure 10), and confirmed that the interfering factors were contained in the membranes. Freeze/thaw supernatants contained material with a similar degree of sulphation to that contained in plasma, serum and standard Opocrin, and may therefore be classed as heparan sulphate or chondroitin sulphate E as before. The material detected in the resuspended cell pellet fraction however, behaved in the same way as Triton X-100 lysates in the assay, and was therefore not comparable with standard Opocrin. The amount of GAG detected in the supernatants from such preparations was relatively low (125-800ng/ml), and did not exceed that in serum (860-3850ng/ml). Moreover, there was high variation with different preparations, and between preparations obtained from the same donor. This method was therefore not appropriate for the determination of total GAG contained in whole blood samples. It was however useful in identifying the exact location of the interfering components in Triton X-100 lysates.

Most chondroitin sulphate molecules (with the exception of chondroitin sulphate E and other variants found in non-mammalian

species), have relatively low degrees of sulphation, and therefore produce a dilution curve with a more horizontal slope in the assay (Figure 7). Since Triton X-100 lysate dilution curves are also relatively horizontal, the possibility that chondroitin sulphate was being detected in these samples was tested (Figure 11). The digestion of chondroitin 4-sulphate by chondroitinase ABC was not impaired in the presence of Triton X-100 (Figure 11a), and chondroitin sulphates naturally occurring in Triton X-100 lysates would therefore have been readily degraded with this treatment. Chondroitinase ABC reduced the apparent concentration of GAG in the lysate samples to some extent, but dilution curves of lysate digests remained non-parallel with standard Opocrin (Figure 11b). This partial loss of assayed material indicates that chondroitin sulphates contained in Triton X-100 lysate samples may have caused some, but not all, the interference detected from these samples in the assay. The majority of the interference was still present after chondroitinase ABC digestion.

Membranes consist mainly of proteins and lipids, and these components were abundant in Triton X-100 lysate samples. Heparin-like polysaccharides have been found to bind directly with lipoproteins contained in whole blood, to form insoluble complexes (Srinivasan et al 1970; Srinivasan et al 1975). Moreover, heparan sulphate is thought to be an essential component of the lipoprotein-lipase binding site of endothelial cell surfaces (Shimada et al 1981; Cheng et al 1981). Lipoproteins bound to GAGs may alter their response in the assay, and this possibility was tested by the removal of lipoprotein from samples by digestion with lipoprotein lipase. Triton X-100 lysate samples treated with this enzyme did not differ from untreated lysate samples when assayed

(Figure 12). Membrane lipids include glycolipids, cholesterol and most abundant are phospholipids. Removal of the phospholipid component in Triton X-100 lysate samples was obtained by digestion with phospholipase C (Figure 13). GAG detected in the lysate samples treated in this way was readily assayed with dilution curves parallel to standard Opocrin; a strong indication that the material detected was similar to that of other biological samples tested. Predigestion of Triton X-100 samples with phospholipase C eliminated much of the interference in the assay. The interfering nature of the phospholipids dissolved in the lysate samples may be explained by their direct binding with GAGs, reducing their Polybrene binding affinity. Moreover, the structure of phospholipids is such that direct competition with free GAG molecules for Polybrene binding sites in the assay may occur.



Basic structure of phospholipids.

Predigestion of lysate samples with phospholipase C introduced a further pretreatment stage, and reproducibility of individual samples in the assay was poor. It was also an expensive pretreatment and therefore was not appropriate for routine use; methods were tested which allowed the total release of GAG from cells, and also removed the interfering components.

The dilution of whole blood samples with Tween 20 followed by centrifugation effectively removed the interfering components (Figure 14), but did not allow the detection of total GAG. This problem was encountered with all methods involving removal of membrane fractions before assay, and attempts to resolve it were the subject of much time and effort, though ultimately ineffective.

Much of the cellular GAG must therefore be contained in, or bound to the membrane component, and some of this is apparently released on clotting. Alternative methods were unsuccessful for similar reasons, and due to lack of time, this problem remained unresolved. Nevertheless, the importance of such values was not ignored, and where necessary, alternative values were used to demonstrate the magnitude of a response. This is discussed in greater detail, and is applied mostly to Chapter 6.

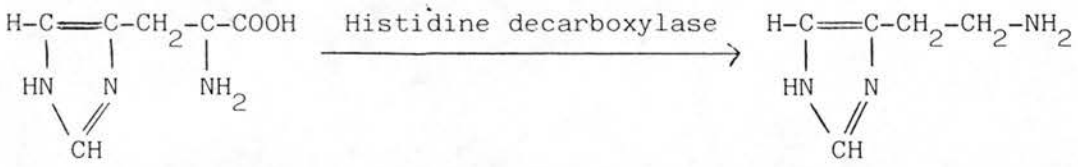
Chapter 2.

(2:2). Measurement of Histamine.

(1). Introduction.

Histamine (β -imidazolylethylamine) is a low molecular weight (111.11) biogenic amine, commonly present in the tissues of mammals and other animals. It is synthesised in mast cells and basophils from L-histidine by the action of L-histidine decarboxylase (Figure A), (Schayer 1963; Galli and Dvorak 1975; Galli et al 1976). This reaction requires pyridoxal-5-phosphate as a cofactor.

Figure A.



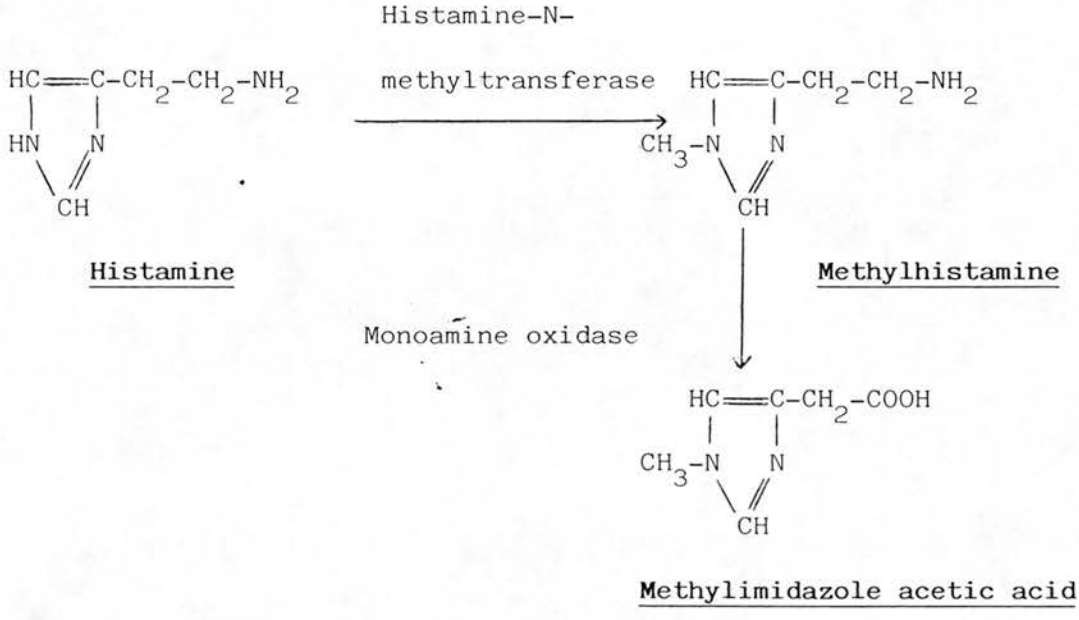
L-Histidine **Histamine**

After synthesis, histamine is thought to be largely confined to the secretory granules of mast cells (Anderson and Uvnas 1975) and basophils (Pruzansky and Patterson 1967), where it associates by ionic interaction with acidic groups of glycosaminoglycans and/or proteins (Uvnas et al 1970). Normal mast cells generally contain more histamine (1-15μ g/10^6 cells), (Patterson et al 1976; Schwartz and Austen 1981), than basophils (1-3μ g/10^6 cells),(Ishizaka et al 1983; MacGlashan and Lichtenstein 1980), although this varies considerably depending on the maturation, nutrition and activation history of the cells.

Nearly all tissues can catabolise histamine (Schayer and Reilly, 1974) and under normal conditions it is metabolised slowly with an

intracellular half-life of approximately 50 days (Schayer 1952). In man, the catabolic route usually involves methylation by histamine-N-methyltransferase to form 1-methyl-4-(ß -aminoethyl) -imidazole (methylhistamine), which is then deaminated to 1-methylimidazole-4-acetic acid by monoamine oxidase (Figure B).

Figure B.

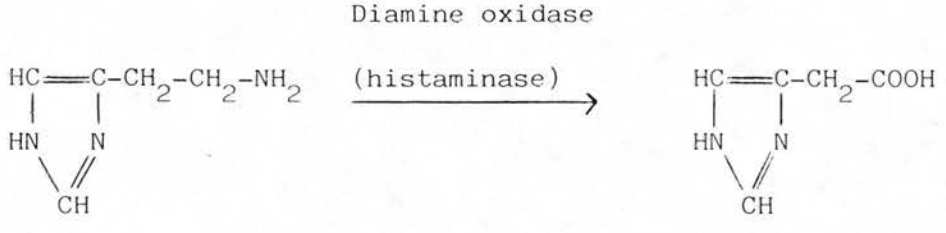


Histamine **Methylhistamine**

Methylimidazole acetic acid

Alternatively, (Figure C), histamine is oxidized to imidazole-4, 5-acetic acid by diamine oxidase (histaminase), which is then largely conjugated and excreted as 1-ribosyl-imidazole-4-acetic acid (Schayer 1959).

Figure C.



Histamine **Imidazole acetic acid**

The biologic response to histamine in the body is mediated by its binding to, and activation of, two different types of tissue or cell receptor, designated H_1 (Ash and Schild 1966) and H_2 (Black et al 1972). The ratio of distribution of these receptors in various organs in the body is not constant (Black et al 1972; Chand and Eyre 1975), but in general contraction of bronchial and gastrointestinal smooth muscle and enhancement of the inflammatory reaction occur via H_1 receptors, whereas stimulation of gastric acid secretion by parietal cells and inhibition of the inflammatory reaction are H_2 receptor mediated (Lichtenstein 1976). Moreover, the stimulation of the two receptor types generally causes antagonistic effects (Brimble and Wallis 1973; Turker 1973).

Histamine can regulate the IgE-mediated release of materials from human basophils (Lichtenstein and Gillespie 1973; Lichtenstein and Gillespie 1975; Lichtenstein 1976) and lung tissue (Chakravarty 1959), as well as lysosomal enzyme release from neutrophils (Zurier et al 1974). It also modulates the function of certain thymus-derived lymphocytes (Lichtenstein 1976; Plaut et al 1974; Roszkowski et al 1977), via H_2 receptors contained on the surface of these cell types. Both H_1 and H_2 receptors on the surface of eosinophils and neutrophils are under the control of histamine, and this regulates migration of the cells in tissues (Clark et al 1975). Thus histamine is essential to the regulation of the inflammatory process.

Histamine may be measured by several techniques. These include:

a. Biological assays, using isolated organs such as the cat or guinea pig terminal ileum in organ bath (Austen 1976), which are very laborious and non-specific, but are however very sensitive.

b. Colorimetric assays following treatment with a diazonium salt

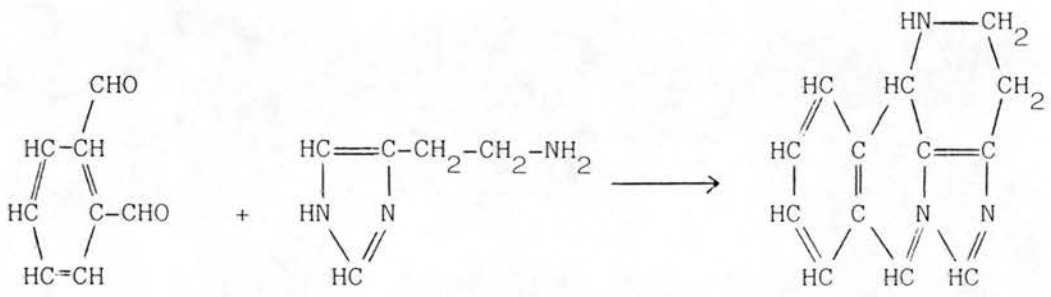
(Rosenthal and Tabor 1948) or dinitrofluorobenzene (McIntire et al 1950; Lowry et al 1954), which are very insensitive.

c. Radioenzymatic assay (Snyder and Taylor 1976), which is specific and sensitive, but requires a very demanding and difficult pig brain enzyme extraction.

d. Fluorometric method (Shore 1976), which requires the separation or extraction of the histamine from samples, but is sensitive and precise.

This fluorometric method was originally described by Shore et al (1959), and has since been improved to increase both specificity and sensitivity (Redlich and Glick 1969; Hakanson et al 1972; Shore 1976). The method is based on the coupling of histamine to o-phthalaldehyde (OPT) at a highly alkaline pH to form a fluorescent product (Figure D).

Figure D.



o-phthalaldehyde **Histamine** **OPT-histamine fluorophore**

Some compounds are known to contribute to the fluorescence observed in the final o-phthalaldehyde reaction (Shore 1971; Siraganian 1974), resulting in falsely elevated values for histamine concentrations. These interfering substances include spermidine, spermine, histidine, arginine, histidyl-histidine, agmatine, reduced glutathione and ammonia at concentrations of greater than 4μ g/ml (Lorenz et al 1970; Shore et al 1959). This problem is

eliminated by the extraction procedure, since approximately 85-92% of available histamine was extracted into the n-butanol (Shore et al 1959) while all other amines remained in the aqueous phase. The histamine was then returned to the aqueous phase, and condensed with OPT in a strongly alkaline solution. The resulting fluorescent product was rather labile, but stability was increased (for up to 1 hour) upon acidification. The formation of the histamine-OPT fluorophore has very specific structural requirements, since alkylation of either of the nitrogen atoms of the imidazole ring or side chain of the histamine molecule inhibits the reaction. The fluorescence intensity of the fluorophore is directly proportional to the histamine concentration over the range 5.0-500ng/ml (Shore et al 1959).

The fluorescence of the histamine-o-phthalaldehyde complex has greater stability and intensity than the complex formed by some other amines. The extraction and assay of histamine by this method is precise, sensitive to concentrations as low as 5ng/ml, and is specific for the quantitative estimation of histamine in body fluids and tissues.

(2). Materials and Methods.

Materials.

Reagents.

Methanol 'AnalaR'(Product code 101586B), n-butanol 'AnalaR'(Product code 100616J), n-heptane 'AnalaR'(Product code 103636C), and perchloric acid 60% 'AnalaR'(Product code 101754W) were all purchased from BDH Chemicals Ltd, Poole, Dorset, England. Histamine dihydrochloride, (crystalline; Product No H7250), o-phthalaldehyde (Product No P0657) and phosphoric acid (Product No P7891) were

purchased from Sigma Chemical Co Ltd, Dorset, England.

OPT Reagent.

10mg of recrystallised o-phthalaldehyde was dissolved in 5ml of methanol, to obtain a solution of 0.2% (w/v). This methanolic solution was stable for approximately two weeks when kept refrigerated in a dark bottle. However, to ensure maximum accuracy, this reagent was made as required.

Methods.

Preparation of Biological Samples.

Protein was removed from samples by perchloric acid precipitation prior to the extraction steps. To 1ml volumes of sample, 0.2ml of 8% perchloric acid was added. These samples were thoroughly mixed, incubated at 37°C for 30 min and then centrifuged (250g; 4°C; 15min), and supernatants retained. The dilution factor introduced at this stage was accounted for when calculating histamine content in each sample.

Extraction of Histamine from Biological Samples.

1ml volumes of perchloric acid treated samples were transferred into 15ml screw-cap glass culture tubes (13 x 100mm with Teflon-lined cap), containing 300mg of NaCl and 1.25ml of n-butanol. To each of these tubes, 0.1ml of 3N NaOH was quickly added and thoroughly mixed for at least 1 min to extract the histamine into the butanol. The tubes were then centrifuged (110g; 4°C; 10min). 1ml of the butanol (top) layer was removed from each tube, and transferred to a 15ml screw cap glass tube containing 1.9ml of n-heptane, and 0.6ml of 0.12N HCl before shaking for at least 1min, and leaving to stand for approximately 10min to allow

for the clear separation of the two phases. 0.5ml of the aqueous phase (bottom layer) was carefully removed from each tube, ensuring that none of the organic phase was transferred at the same time. These samples were then assayed fluorometrically as described below.

Histamine Standards.

A stock solution containing 10µ g/ml of histamine dihydrochloride was prepared, using 0.12N HCl as the diluent, and stored in 0.5ml aliquots at -20°C. Standards containing 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01µ g/ml in 0.12N HCl formed the standard curve. Blank control samples (containing 0.12N HCl only) and quality control samples (containing 40 and 80 ng/ml of histamine dihydrochloride were included in each assay.

All standards and samples were prepared for assay in duplicate.

Fluorometric Assay.

To all tubes, containing 0.5ml of standard or sample, 0.1ml of 1N NaOH was added to provide the strongly alkaline conditions required for the condensation of histamine with OPT. This was followed by the addition of 25µ l of OPT, thorough mixing, and transfer to an ice bath (approximately 4°C). The reaction was allowed to proceed for 40min and was stopped by adding 50ul of 2M phosphoric acid (H_3PO_4) to each tube. The tubes were thoroughly mixed and allowed to equilibrate to room temperature for approximately 15min before reading on a Perkin Elmer Fluorescence Spectrometer with an activation wavelength of 363nm and a fluorescence wavelength of 468nm.

Reversed blanks were used to ensure there was no non specific

binding of histamine with OPT under less alkaline conditions. These were prepared by adding 25µ l of 2M H_3PO_4 to control tubes, before the fluorescent label.

(3). Results.

Measurement of histamine standard.

Histamine dihydrochloride was diluted in 0.12N HCl, and a typical standard curve is shown in Figure 1. Histamine dihydrochloride standards were assayed, diluted in plasma and Tris ACM buffer (diluent for cell suspensions in release studies; Chapter 6), and compared with the normal standard diluent of HCl, to determine any interference they may cause in the assay. All standard curves produced were identical.

Effect of perchloric acid on histamine standard.

Histamine dihydrochloride standards were assayed, diluted in 1.5% and 3% perchloric acid solutions. Using 1.5% perchloric acid as the assay diluent produced a curve similar to that of the standard, whereas 3% perchloric acid significantly reduced the fluorescence of the OPT-histamine complex (Figure 2).

Measurement of Histamine in Biological Samples.

The histamine content in each of the biological test and control samples, was determined by direct comparison with the standard curve to give mass histamine (values of histamine dihydrochloride/ml). This value was then converted to true histamine/ml by a conversion factor of 0.603, calculated as follows:

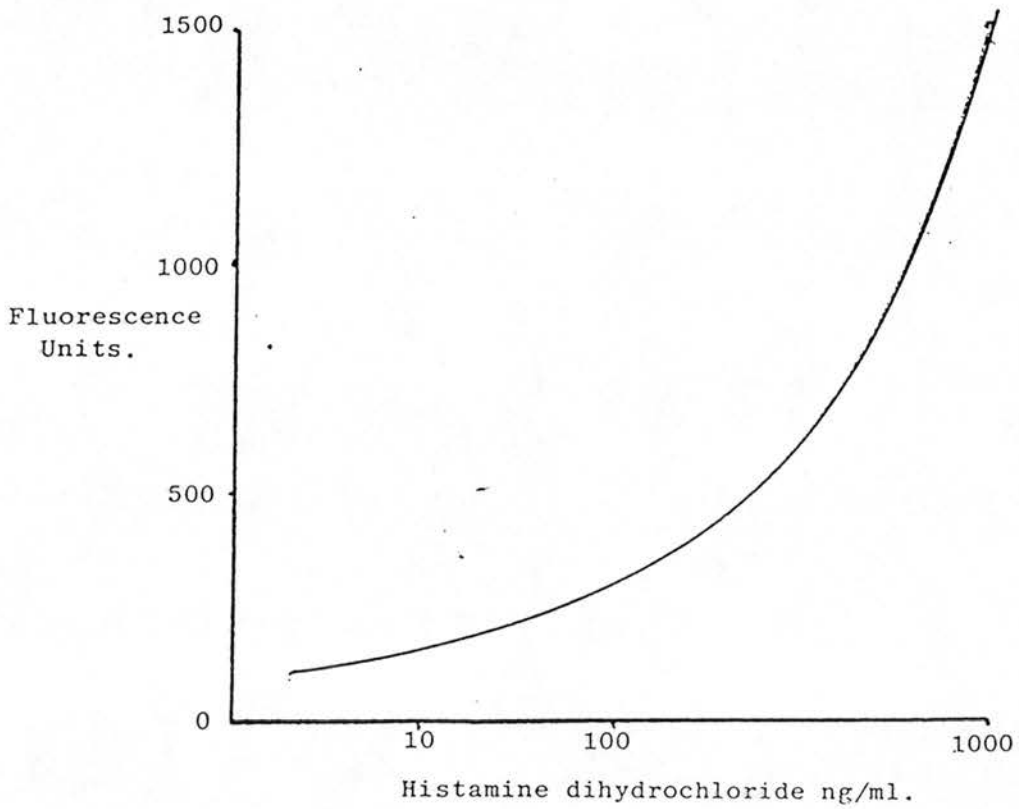


Figure 1: Example of a Typical Histamine Standard Curve.

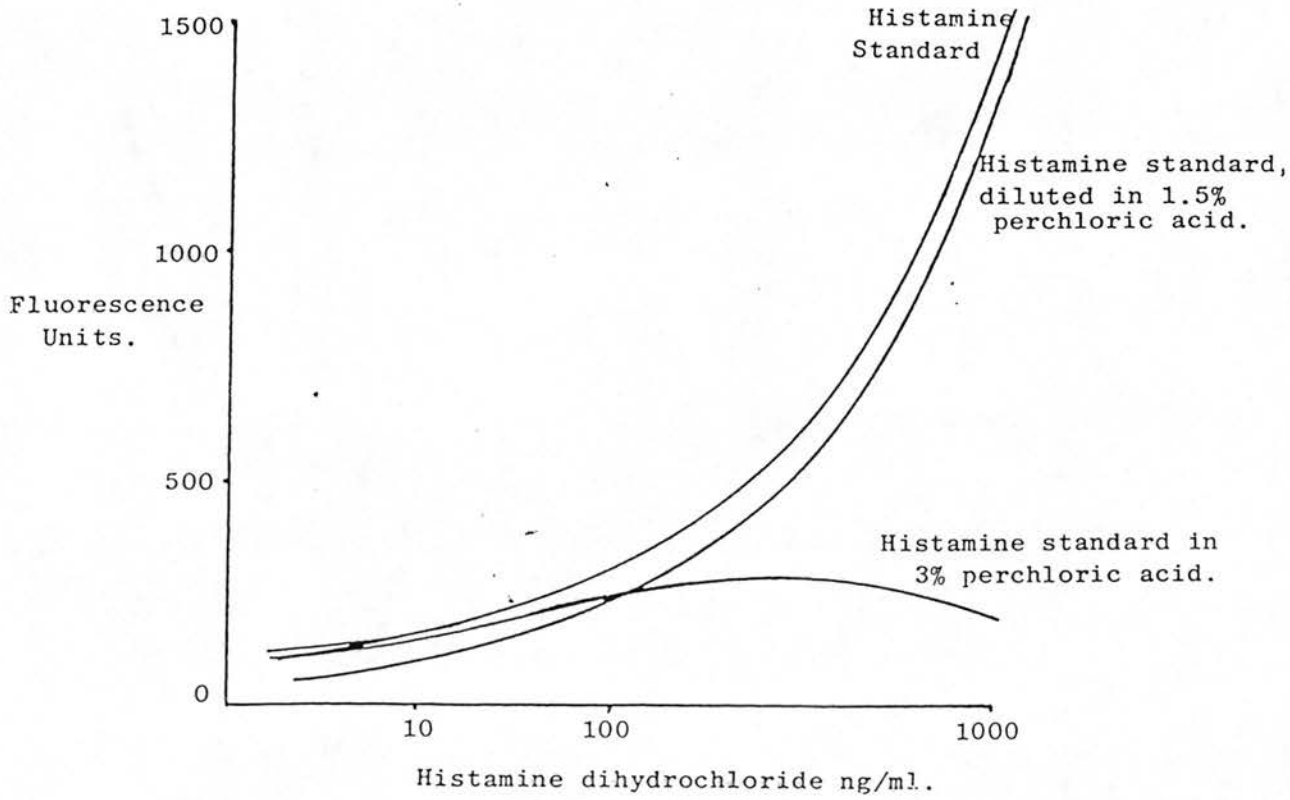


Figure 2: Effect of Perchloric Acid on Histamine Standard Curve.

Using 1.5% perchloric acid as assay diluent did not affect the standard histamine, whereas 3% perchloric acid significantly reduced the fluoresence of the OPT-histamine fluorophore.

Molecular weight of histamine = 111.11.

Molecular weight of histamine dihydrochloride = 184.11.

$$\text{Histamine moiety in sample} = \frac{\text{Mass histamine} \times 111.11 \text{ ng/ml.}}{184.11}$$

$$= \underline{\textbf{Mass histamine x 0.603 ng/ml.}}$$

The intra-assay Coefficient of Variation (CV) for quality control samples containing 40 and 80 ng/ml of histamine dihydrochloride was 10.7% and 10.3% respectively. The inter-assay CV for samples containing 25 and 75ng/ml of histamine dihydrochloride was 10.6% and 12.9% respectively.

Activation and Fluorescence Spectra of the OPT Fluorophore.

The activation and fluorescence spectra of the OPT fluorophore were determined by testing its fluorescence on variable activation and excitation wavelengths, using a scanning fluorometer (Figures 3a and 3b).

a. Activation of fluorophore.

The exitation or fluorescence monochromator of the scanning fluorometer was set at 450nm, and the spectrum from the activating monochromator was scanned between 300 and 400nm. Figure 3a shows the spectrum obtained; maximum activation of this fluorophore occurs at 350nm.

b. Fluorescence (or exitation) of fluorophore.

The activating monochromator was set at 350nm, and the spectrum from the excitation monochromator was scanned between 300 and 600nm. Figure 3b shows the spectrum obtained, with maximum fluorescence at 450nm.

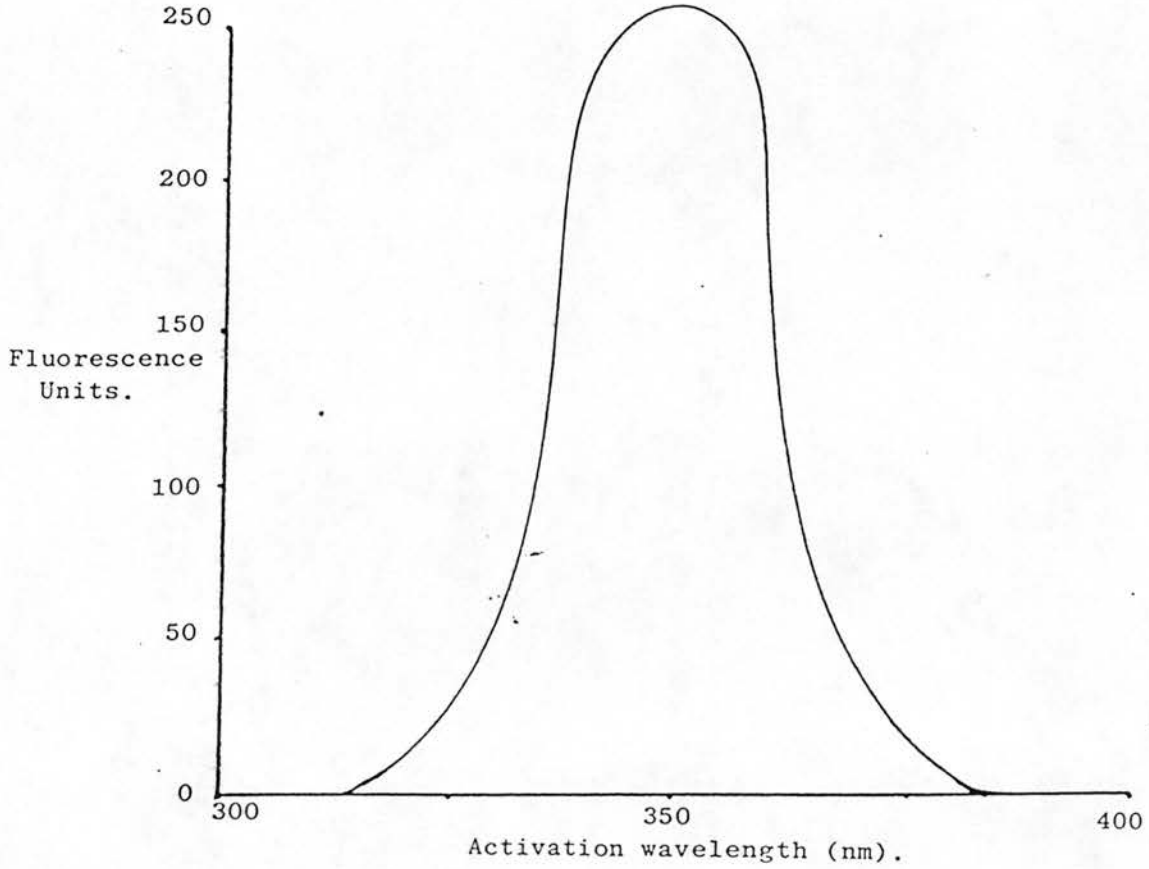


Figure 3a: **Spectrum of Activation of Fluorophore.**

The maximum activation of the OPT-histamine fluorophore occurred at 350nm.

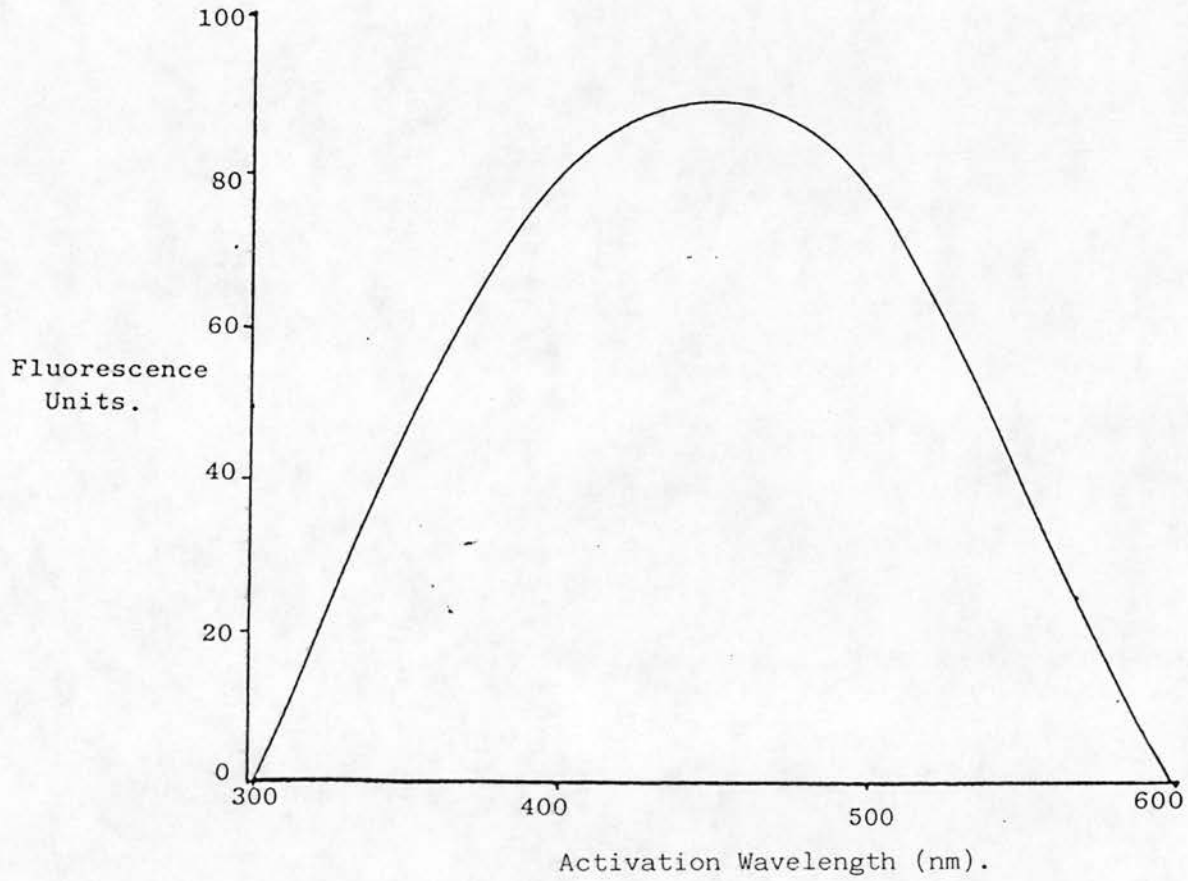


Figure 3b: **Spectrum of Fluorescence (or Excitation) of Fluorophore.**

The maximum fluorescence or excitation of the OPT-histamine fluorophore occurred at 450nm.

This procedure was repeated using a sample containing a large concentration of histamine (95ng/ml). The peaks obtained for both spectra occurred at the same wavelength as those of the standard histamine-OPT fluorophore. The activation and fluorescence spectra of the OPT fluorophore used in this assay (Figures 3a and 3b) produced the same pattern as those originally obtained by Bowman et al (1955).

% Efficiency at Filter Wavelengths.

The Perkin Elmer Fluorescence Spectrometer used to determine fluorescence routinely had fixed wavelength filters of 363nm (activation), and 468nm (fluorescence). Since these were not the wavelengths at which maximum emission of the fluorophore occurred, the efficiency of these filters (expressed as % maximum) was determined, using the activation and fluorescence spectra as follows:-

a. Activation wavelength efficiency.

$$\text{Activation wavelength efficiency} = \frac{\text{Emission at 363nm} \times 100}{\text{Emission at 350nm}}$$

$$= \frac{215.1 \times 100}{225.0}$$

$$= \underline{\textbf{95.6\% of maximum.}}$$

b. Fluorescence wavelength efficiency.

$$\text{Fluorescence wavelength efficiency} = \frac{\text{Emission at 468nm} \times 100}{\text{Emission at 450nm}}$$

$$= \frac{82.8 \times 100}{86.5}$$

$$= \underline{\textbf{95.7\% of maximum.}}$$

(4). Discussion.

It is generally accepted that in humans all blood histamine is associated with basophils (Erlich 1953; Graham et al 1955; Valentine et al 1955) and is confined largely to their specific cytoplasmic granules. Studies on basophil degranulation have revealed that nearly all this histamine is released on immunological stimulation (Lichtenstein 1968). Using the fluorometric method for the detection of histamine, the total histamine content of whole blood was found to be 83ng/ml (SD=2.45ng/ml; range=32.4-173.5ng/ml), and histamine release from whole blood correlates with its release from washed leucocyte preparations (r=0.93) (Siraganian and Brodsky 1976). On the basis of these observations, the fluorometric assay was utilised for the study of histamine release from basophil enriched fractions of whole blood (Chapter 6). Although the sensitivity of this assay is not sufficient to detect plasma histamine levels, it is nevertheless adequate for the requirements of this study. Using a more sensitive enzymatic method for histamine determination in human plasma, the average normal concentration was found to be 0.69ng/ml, SD=0.26ng/ml (Lorenz et al 1972). However, widely discrepant results were revealed by a survey of 19 laboratories, using similar methods for the detection of histamine in human plasma (Gleich and Hull 1980). The levels of histamine in plasma are very low, and therefore difficult to determine accurately. Moreover, there may be rapid fluctuation due to the influence of many external factors including cold, and physical stimuli such as exercise. As the interpretation of plasma levels is open to question, no attempt was made to increase the sensitivity of this assay for the detection of histamine in plasma.

The manual extraction and assay of histamine by the fluorometric method is accurate and sensitive for the determination of this biologically active molecule over the range 5.0-500ng/ml (Shore 1959). It was necessary to remove protein from samples, and the standard method of perchloric acid precipitation (Siraganian and Hook 1976b) was used. Samples were therefore pretreated by diluting 5:1 (vol sample/vol acid) with a solution of 8% perchloric acid, and centrifuging to remove precipitates. Histamine standards did not contain significant amounts of protein to warrant this pretreatment, but any interference it may cause was tested on the standard curve. Final concentrations of 1.5% perchloric acid or less did not interfere with fluorescence of the fluorophore formed by standard histamine. This was important, since the final concentration of perchloric acid used to treat the biological samples was 1.33%, and it was therefore concluded that this pretreatment did not interfere with measurement of the histamine contained in samples.

Since the OPT reagent is itself fluorescent, its contribution to the final fluorescence obtained from samples after assay was determined in each assay, by using reversed blanks as controls. In these control samples, the condensation step was inhibited by introducing acid conditions before the addition of OPT. Reagent blanks were used as a second internal control for each assay, and if values obtained from these tubes were more than 10% higher than the reversed blanks, the assay was considered invalid.

The fluorescent and exitation ability of the fluorophore produced by the biologically occurring histamine was studied and compared with that of standard histamine. The fluorescence and exitation spectra obtained from both samples were identical, validating the

use of this standard, for quantitation of the histamine naturally occurring in biological samples. The filters used to determine the fluorescence of standards and samples in each assay were not of the wavelengths at which maximum fluorescence and emission of the fluorophore occurred. They were nevertheless able to detect significant fluorescence with sufficient accuracy for this method, and were therefore used consistently throughout the study.

Chapter 3.

Characterization of Glycosaminoglycans.

(3:1). Introduction.

Glycosaminoglycans (GAGs) are a ubiquitous component of mammalian cells, mostly occurring as cell surface-associated proteoglycans (Kraemer 1971a; Kraemer 1971b). GAGs are widely distributed in animal tissues; different cell types and species synthesize and contain different classes of GAG, and this has been the subject of much research in recent years. Heparin is a possible constituent of blood vessels (Haruki and Kirk 1964; Stevens et al 1977; Castellot et al 1981), and has been reported to be present in circulating blood (Eiber and Danishefsky 1957; Horner 1974), and in plasma, but is not freely available (Staprans and Felts 1985). However the majority is associated with mast cells (Jorpes et al 1937; Straus et al 1982). Heparin has been isolated from rat skin (Horner 1971) and from rat peritoneum (Yurt et al 1977), and human tissue (Metcalfe et al 1979; Metcalfe et al 1980), and it has been proposed that all physiologically available heparin occurs exclusively in the granules of these mast cells (Scully et al 1986). However, some mast cells contain other GAGs; cultured purified rat peritoneal mast cells have been found to synthesize proteoglycan with chondroitin sulphate side chains posessing large amounts of N-acetylgalactosamine-4,6-disulphate (Czarnetzki and Behrendt 1981; Stevens and Austen 1982;) rather than heparin. Studies revealed that in these rodent mast cells, the chemical composition of proteoglycan synthesized changes as the cells go through a transition during maturation, from having GAGs of

relatively low sulphation, comparable with chondroitin sulphate, to highly sulphated GAGs, corresponding to heparin or chondroitin sulphate E. Typically, mature mast cells grown from primary mouse embryonic fibroblast monolayers were found to contain 20-40% chondroitin sulphates, and 60-70% heparin (Bland et al 1982), whereas cultured bone marrow-derived mucosal mouse mast cells contained chondroitin sulphate E, but not heparin (Razin et al 1982).

The synthesis of GAG in mature serosal mast cells was found to be influenced by the presence of p-nitrophenyl-ß -D-xyloside (Stevens and Austen 1982), which stimulated the production of chondroitin sulphates A, C and E, as well as heparin. Moreover, preferential synthesis of chondroitin sulphate in mouse mastocytoma cells, normally synthesising both chondroitin sulphate and heparin (Lewis et al 1973) occurs in the presence of this xyloside (Robinson and Lindahl 1981). In general the composition of GAG in serosal and mucosal mast cells follow specific patterns, which may be altered during cell maturation and chemical stimulation. The chemical composition of the GAG synthesised in a particular mast cell is one feature that has been used to define mast cell heterogeneity, and various other differences observed between mast cells from the mucosal surface and those present in the peritoneal cavity have been well documented (Enerback 1981; Bienenstock et al 1982; Pearce 1983). The existence of these two mast cell subsets in rodents highlight the similarities between mucosal mast cells and basophils (Gleich et al 1986; Schwartz 1986), and has been discussed in Chapter 1.

Differences between human intestinal mucosa and lung parenchyma mast cells have not been detected by either morphologic or electron

microscopic studies (Fox et al 1985), and Lichtenstein et al (1986) have been unable to identify these two mast cell subsets in humans, although human mast cells are heterogeneous (Schulman et al 1983; Shanahan et al 1987). Mast cells in general have many biochemical and morphological similarities with basophilic leucocytes, and are thought to arise from the same progenitor (Zuker-Franklin 1980). One common feature is in their staining properties, and it was assumed that the GAG responsible for taking up the stain was the same as in mast cells; heparin. However on the basis of cytochemical studies, Hakomori (1965) studied this further and suggested that hyaluronic acid, heparin or heparan sulphate is present in granules of these cells. Later Olsson et al (1970) demonstrated the presence of a heparin-like substance in human basophilic leucocytes. Material was classed as being 'heparin-like', as it contained both sulphate and glucosamine residues, and was resistant to degradation by hyaluronidase, but sensitive to heparinase. A second class of GAG was also detected which contained galactosamine residues, and was classed as chondroitin sulphate. However, density gradient centrifugation revealed that only the heparin-like substance followed the distribution of basophils. These data, and earlier reports of the presence of chondroitin sulphate in neutrophilic granulocytes (Olsson and Gardell 1967), indicated that chondroitin sulphate may be present in various blood cell types, but that heparin-like GAG was exclusively contained in basophilic leucocytes.

Recent reports of GAG isolated from basophils, have been of chondroitin monosulphates or oversulphated chondroitin sulphate E (Metcalfe et al 1984; Seldin et al 1986; Rothenberg et al 1987), and the glucosamine/sulphate rich moiety isolated by Olsson et al

(1970) seems to have been ignored. The aim of the studies described in this chapter was to determine the cellular location and biochemical nature of GAG contained in whole blood.

(3:2). Materials and Methods.

Materials.

Reagents.

Human serum albumin was purchased from KabiVitrum Ltd, London, UK, and hydroxychloroquine sulphate from Sterling-Winthrop, Surbiton-upon -Thames, Surrey, UK. Lymphoprep and Ficoll-metrizoate were purchased from Nyegaard, Oslo, Norway, and Dextran T-500 from Pharmacia (GB Ltd), Milton Keynes, England. Fractogel TSK HW 55(F) was purchased from Merck, Darmstadt. Benzamidine hydrochloride (Product no B6506), 6-aminohexanoic acid (E-amino-n-caproic acid; Product no A2504), heparitinase (Product no H6512) and chondroitinase ABC (Product no C2905) were all purchased from Sigma Chemical Company Ltd, Dorset, England.

Buffers.

Tris A.

This buffer was first used in studies with leucocytes by Lichtenstein and Osler (1964), and consisted of:

0.025M Tris-(hydroxymethyl)-methylamine (Tris).

0.12M NaCl.

0.005M KCl.

0.3mg/ml human serum albumin.

This was adjusted to pH 7.6 at room temperature with 1N HCl.

Washing buffer.

This consisted of Tris A buffer with hydroxychloroquine added to a

concentration of 5mM.

Moore and James Counting Stain.

This consisted of:

40ml 0.05% Toluidine blue in 0.85% NaCl.

11ml 95% Ethyl alcohol.

1ml Saturated solution of saponin in 50% ethyl alcohol.

Fractogel buffer.

This consisted of:

0.05M Phosphate buffer (pH 7.4).

0.2M NaCl.

3mM Sodium azide, (NaN_3).

Citrate buffer.

This consisted of:

13mM Trisodium citrate.

3mM Sodium azide, (NaN_3).

This was adjusted to pH 6.7 at room temperature with 1N HCl.

Methods.

Blood Fractionation Methods

Cellular fractions from whole blood were obtained by density gradient centrifugation, and these were performed by Dr IR MacGregor. The methods used were as follows.

Basophils.

Basophils were separated from peripheral blood based on the method of Day (1972). Whole blood was taken into ETP anticoagulant (described in Chapter 2), in an ice bath, and 10ml of anticoagulated blood mixed with 10ml of 0.9% w/v NaCl, and layered over 10ml Lymphoprep in a polycarbonate centrifuge tube. After centrifugation with a swing out head (400g at the interface; 30

min; room temperature), supernatant down to 2mm from the red cell pellet was removed, mixed gently, and layered over cold buffered Ficoll-metrizoate solution with a density (d) of 1.065g/ml. Hydroxychloroquine was added to all density solutions at a final concentration of 5mM to stabilise cell membranes, and thereby reduce release reactions during fractionation. The tube was centrifuged (400g; 15min; room temperature) and the platelet-plasma layer and the separating fluid (down to the cell pellet) was removed and discarded. The basophil-enriched cell pellet was resuspended in washing buffer, and after centrifugation (100g; 5min; room temperature) the supernatant was removed, and the cell pellet resuspended in 2.5ml of Ficoll-metrizoate with d=1.085. A gradient was formed using this and an equal volume of buffer with d=1.065 in an 8ml centrifuge tube, and centrifuged at 3800g for 30min. Acceleration and deceleration were controlled manually over 5min periods. The supernatant was removed and the pellet was washed twice in washing buffer at 100g for 5min, before final resuspension in 1ml washing buffer. Cell counts were performed using the stain of Moore and James and counted in a Neubauer haemocytometer counting chamber.

Platelets and Erythrocytes.

A 6ml sample of ETP anticoagulated whole blood was centrifuged at 120g for 15min at 4°C. Supernatant platelet-rich plasma (PRP) was removed and centrifuged at 4800g for 15min at 4°C; this pellet was resuspended in 6ml of washing buffer and centrifuged at 2000g for 15min at 4°C. The platelet pellet was finally resuspended in 2ml washing buffer.

The red blood cell pellet retained from the initial centrifugation stage, plus trapped plasma, was resuspended in washing buffer, and

centrifuged (4800g; 15min; 4°C). Plasma supernatant was removed, and the red cell pellet resuspended in 6ml of washing buffer and centrifuged (2000g; 15min; 4°C). The cell pellet was finally resuspended in 10ml of washing buffer.

Lymphocytes.

A 9ml sample of ETP anticoagulated blood was diluted with an equal volume of 0.9% (w/v) NaCl solution at room temperature, and layered on 9ml of Lymphoprep and centrifuged (800g; 15min; room temperature). Cells at the interface were carefully removed with a Pasteur pipette, without removing the upper layer. 7ml of cell suspension was carefully mixed with 21ml of washing buffer and then centrifuged at 80g for 10min at room temperature. Supernatant was removed and the pellet resuspended in 20ml of washing buffer, centrifuged (80g; 10min; room temperature) and the pellet finally resuspended in 1ml of washing buffer.

Eosinophils and Neutrophils.

The residual red cell-granulocyte pellet from the lymphocyte preparation procedure was resuspended in 1ml EDTA (0.01M) plasma, with 0.4ml of 4.5% (w/v) Dextran T500 in 0.9% (w/v) NaCl solution and kept at 4°C for 3h. Supernatant fluid was removed (0.75ml), and half was layered over 4ml of Ficoll-metrizoate (in distilled water) with a density of 1.195. The remaining supernatant was layered over 4ml of Ficoll-metrizoate with density of 1.148. Both tubes were centrifuged at 400g for 40min at room temperature. The cell pellets, eosinophils for light solvent and neutrophils for dense solvent, were resuspended in 5ml of washing buffer and centrifuged (100g; 10min; room temperature), then resuspended in 1ml of washing buffer. Since a good pellet of neutrophils at d=1.195 was not obtained, the supernatant of d=1.148 was diluted, centrifuged, and this pellet used.

Basophil enriched fractions from peripheral blood.

Basophil enriched fractions from whole blood samples were prepared essentially as described by Siraganian and Hook (1976b). From each individual studied, 40ml of whole blood was taken into 7ml of 2.14% clinical grade Dextran/4.29% dextrose,(w/v)/0.025M EDTA. This was divided equally between two 50ml plastic tubes, which were then placed at an angle of 45°, and allowed to settle for 90min. All subsequent steps were performed at 4°C. Supernatant leucocyte-rich plasma from both samples were carefully withdrawn using a Pasteur pipette, and pooled in a 50ml plastic centrifuge tube. Since basophils were contained in the sediment just above the red cell layer, some of the red cell layer was removed with the plasma, to ensure maximum recovery of basophils. The supernatant plasma was then centrifuged (110g; 15min; 4°C), supernatant removed and the cell pellet resuspended in 0.01M phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl. This was then centrifuged (110g; 15min; 4°C). The supernatant was removed and the cell pellet resuspended to 2ml using 1M NaCl containing:

0.05M sodium acetate,

0.01M EDTA,

0.1M 6-amino-hexanoic acid,

0.005M benzamidine HCl.

Extraction of Glycosaminoglycan.

The cellular suspension was frozen to -20°C and thawed six times, and then dialysed overnight to remove NaCl, with two 1 litre changes in dialysing buffer of 0.05M sodium acetate,

0.01M EDTA,

0.1M 6-amino-hexanoic acid,

0.005M benzamidine HCl.

The dialysate was then centrifuged to remove cell debris (10,000g; 30min; 4°C). The supernatant was carefully removed and prepared for loading onto columns. An internal marker was added to each sample, in the form of control heparin tracer (at 10ng/ml). Glucose was also added (at 10mg/ml) both as an indicator of the column bed volume, and to increase the density of the sample to allow efficient layering on the column.

Determination of Molecular Weight of Glycosaminoglycan.

The molecular size distribution of GAG in samples was compared with that of ^{125}I-heparin, by gel filtration using Fractogel TSKHW-55(F) (150mls) decanted into a 1.6cm x 100cm glass column (Column C16/100; Pharmacia) containing a fixed porous plate. The gel was washed thoroughly with fractogel buffer; immediately before use, the buffer was drained to the interface in the column, carefully ensuring that all the gel was covered. The prepared sample was then layered over the interface without disturbing the packed gel. Fractogel buffer was then carefully layered over the sample, until the column was completely filled. The column was eluted with 200ml of fractogel buffer, and 40 fractions of 5ml volume were collected at room temperature overnight, in 13mmx100mm glass disposable culture tubes (Corning) using a Frac-100 fraction collector (Pharmacia, Milton Keynes, England). Each fraction was counted on a NE 1600 γ -counter to detect the heparin tracer marker. Each fraction was also tested for the presence of glucose using BM test strips (BM Test-7; Cat no 290238, Boehringer Co, East Sussex, England). A sample of each fraction was then treated with Pronase (5mg/ml), and assayed for GAG as described in Chapter 2:1, to determine its elution position.

Determination of Degree of Sulphation of Glycosaminoglycan.

The relative degree of sulphation of GAG in samples was compared with that of ^{125}I-heparin by the elution position obtained from a column of Polybrene immobilised on epoxy-Sepharose 6B (Pharmacia) according to the method described by MacGregor et al (1984), using a gradient of 0-2M NaCl in citrate buffer.

Polybrene-Sepharose (25ml) was decanted into a 25mmx150mm glass column containing a fixed porous plate, and washed thoroughly with citrate buffer. Prepared samples were layered onto the interface, carefully ensuring the Polybrene-Sepharose was not disturbed. Citrate buffer was then carefully layered over the sample, and 20 fractions of 5ml volume were collected. All the sample was eluted at this stage, except for GAG-containing material which remained on the column bound to Polybrene. A 0-2M NaCl gradient in citrate was then used to elute GAGs from the column, and 60 fractions of 5ml volume were collected over this range. A Frac-100 fraction collector was used to collect these fractions overnight in 13mmx100mm glass culture tubes, and each fraction was counted on a NE 1600 γ-counter to determine elution of the heparin tracer marker, and tested for the presence of glucose as before. The salt concentration in every fifth sample was measured conductimetrically. NaCl was removed from these samples by overnight dialysis in 5 litre volume of citrate buffer at 4°C. Each dialysate was then treated with Pronase (5mg/ml), and assayed for GAG as before.

Effect of Protease Digestion.

To assess the effect of proteases on GAG contained in samples, Pronase was added to prepared samples (at 5mg/ml), and incubated overnight at 37°C, before preparing and loading onto columns as before.

Effect of β -Elimination.

Prior to addition to column, the sample was treated with an equal volume of 1M NaOH, overnight at 37°C. The sample was then prepared and loaded onto the column as before.

Effect of Chondroitinase ABC Digestion.

The sample was prepared as previously described; chondroitinase ABC was added at a final concentration of 0.2 units/ml, and incubated at 37°C overnight. The sample was then prepared and loaded onto the column as before. In control experiments to test the activity of chondroitinase ABC, chondroitin 4-sulphate (10mg/ml) was diluted in normal human plasma, and treated with chondroitinase ABC (0.2 units/ml). The elution position of chondroitin 4-sulphate was determined by measuring metachromasia with azure A (Lam et al 1976).

Effect of Heparitinase Digestion.

The sample was prepared as previously described, and dialysed overnight in 0.25M sodium acetate containing 0.025M calcium acetate (pH 6.8). Heparitinase was added to this dialysate at 0.5 units/ml, and incubated at 30°C for at least 3h. After incubation, the sample was prepared and loaded onto the column as before. In control experiments to test the activity of heparitinase, Organon 10172 (10mg/ml) was diluted in normal human plasma and treated with heparitinase as described above.

Effect of Nitrous Acid Treatment.

Samples were added to 0.24M $NaNO_2$ (final concentration) in 1.8M acetic acid solution and adjusted to pH7 using Tris buffer and 4M NaOH. Samples were incubated at 20°C for 80min before assay.

(3:3). Results.

Density Gradient centrifugation.

Cell fractions were isolated by the method of Day (1972), and Table 1 shows the cell count obtained in each preparation. Purity was > 84% in each case. Each cell fraction lysate was assayed for GAG, which was found to be mostly contained in the basophil fraction. The small quantity detected in the lymphocyte fraction was probably due to basophil contamination (Table 1).

Calculation of Molecular Weight of GAG.

The relative elution positions of a number of GAGs of known molecular weight from the Fractogel TSKHW-55(F) column, using ^{125}I-heparin as an internal standard, were used to produce a calibration curve (Figure 1). The molecular size distribution of GAG in each experimental sample was compared with that of the internal marker ^{125}I-heparin in each elution profile, and using the calibration curve, its molecular weight was measured.

The elution profile of the heparan sulphate standard is shown in Figure 2, and using the calibration curve, its molecular weight was estimated at approximately 40,000. The smaller second peak of radioactivity detected in later fractions was due to unbound ^{125}I.

The elution profile in Figure 3 illustrates that the material contained in human basophils covered a wide range of molecular weight, from over 500,000 to 2,300. This was repeated using basophil fractions from two different individuals, and similar results were obtained (Table 2).

Table 1 Concentration of GAG in Various Fractions of Whole Blood following Density Gradient Centrifugation.

	Cell count/ml	GAG HS equiv. $\mu g/10^6$ cells
Erythrocytes	2.3×10^9	0.2
Platelets	146×10^6	0.2
Lymphocytes	4.3×10^6	6.96
Eosinophils	0.02×10^6 (84% eosinophils; 16% neutrophils)	0.39
Neutrophils	0.12×10^6 (84% neutrophils; 16% lymphocytes)	0.39
Basophils	0.23×10^6 (90% basophils; 10% monocytes)	243.3

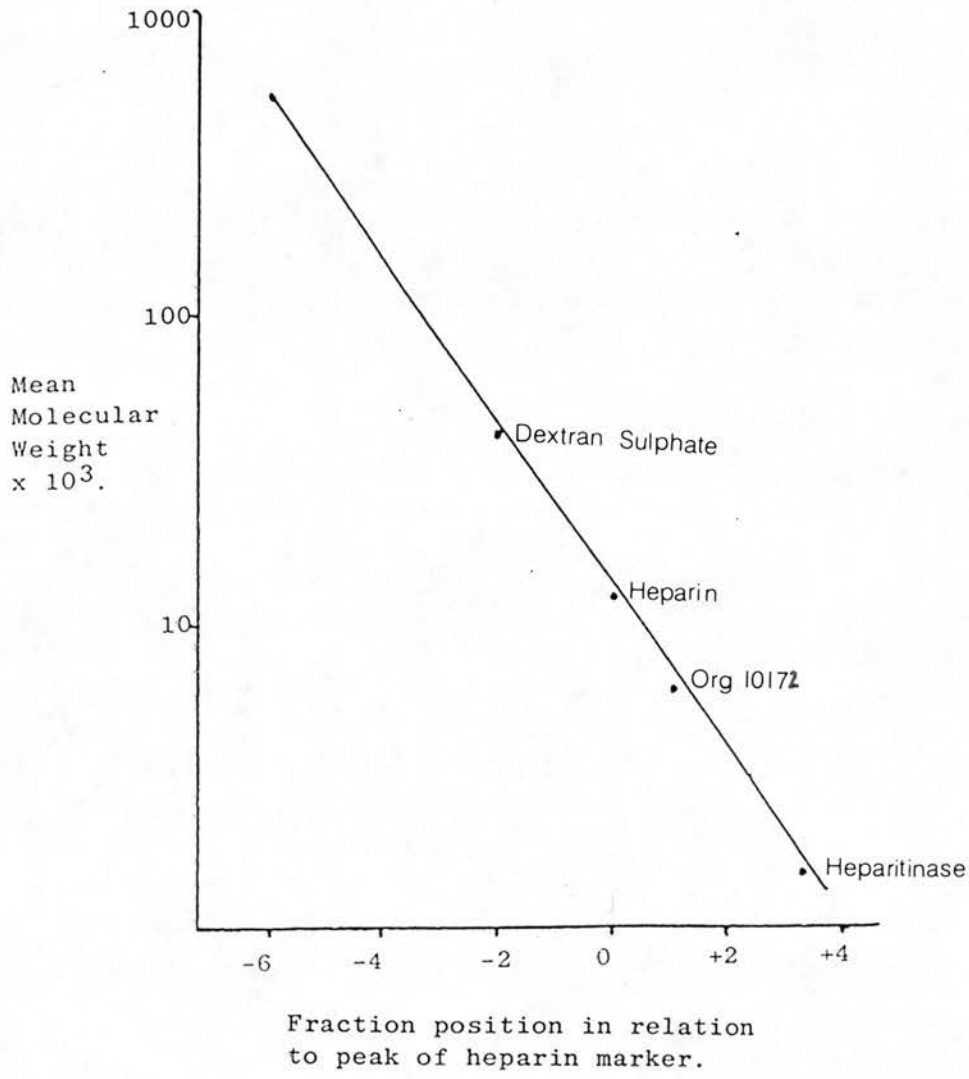


Figure 1. Calibration Curve.

The relative elution positions of a number of known molecular weight GAGs from the Fractogel TSKHW-55(F) column with ^{125}I-heparin were used to form a standard calibration curve.

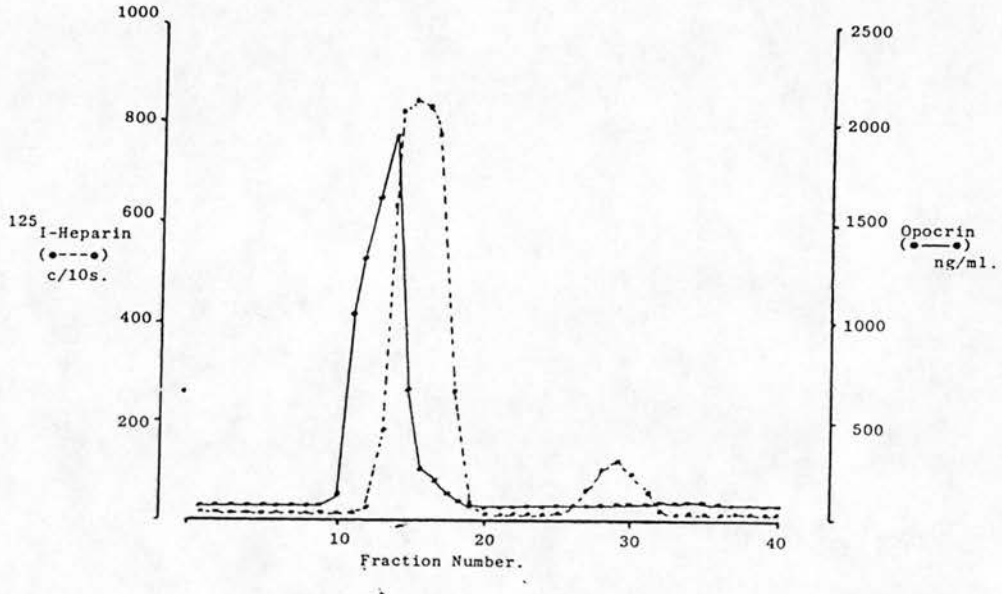


Figure 2. Elution profile from Fractogel TSKHW-55(F) of heparan sulphate standard (Opocrin) (•——•) and ^{125}I-heparin tracer (•---•). The Opocrin eluted at a position equivalent to 40,000 mol wt.

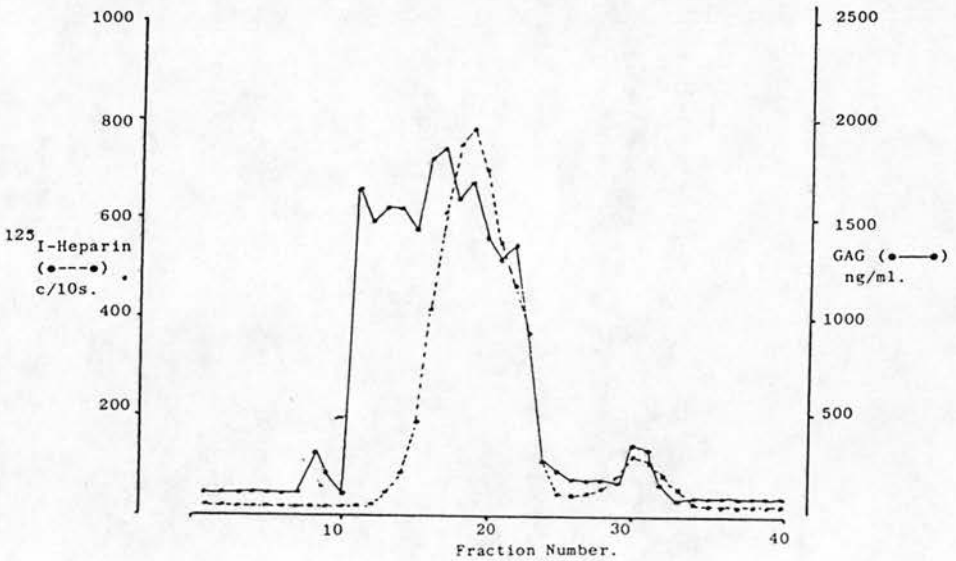


Figure 3. Elution profile from Fractogel TSKHW-55(F) of GAG extracted from a basophil enriched fraction of whole blood (•——•) and ^{125}I-heparin tracer (•---•). The sample material had a wide range of high molecular weight GAGs, from 2,300 to over 500,000.

Table 2: Size of Basophil-Derived GAG.

	Molecular Weight Range.
Individual 1	2,300 - > 500,000.
Individual 2	2,000 - 220,000.
Individual 3	2,500 - 270,000.

Determination of Degree of Sulphation of GAG.

Heparan Sulphate Standard.

The NaCl concentration at which a GAG was eluted from the Polybrene-Sepharose column, varied according to degree of sulphation, and the relative degree of sulphation of GAG in each sample was therefore determined by comparing its elution pattern with that of the internal marker, ^{125}I-heparin. The elution profile in Figure 4 illustrates that the heparin control was displaced from the Polybrene-Sepharose column when the concentration gradient reached 1M NaCl. Less sulphated GAG molecules were contained in earlier fractions, since they had lower affinities for Polybrene, and therefore elution occurred at lower concentrations of NaCl. Heparan sulphate standard was eluted from the column over the concentration range 0.62-0.80M NaCl. The peak of radioactivity detected in the first 20 fractions was due to unbound ^{125}I.

GAG released from human basophils.

Material released from human basophils (taken from the same individual as in Figure 3) was also contained in earlier fractions than the heparin standard, and was eluted from the Polybrene-Sepharose column over the concentration range 0.53-0.73M NaCl (Figure 5).

This supported earlier findings (Chapter 2:1) that the material contained in basophils was of a similar degree of sulphation to that of heparan sulphate standard. This was repeated using basophil fractions from two different individuals, and similar results were obtained (Table 3).

GAG contained in human plasma.

40ml of citrated plasma was tested on the Polybrene-Sepharose column, and material was eluted at 0.55-0.71M NaCl. Again this was

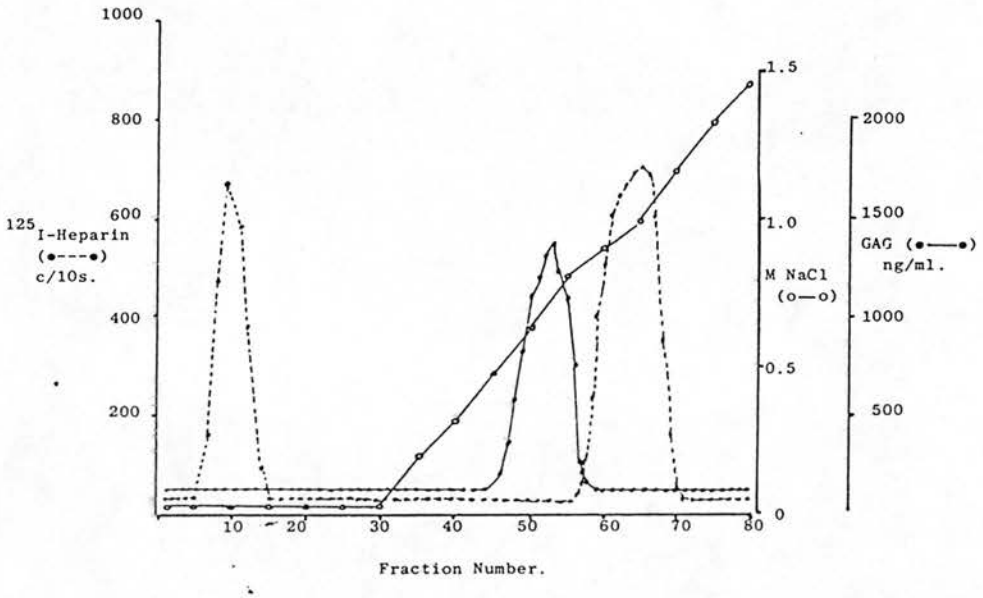


Figure 4. Elution profile from the Polybrene-Sepharose column with 0-2M NaCl gradient (o——o) of heparan sulphate standard (•——•) and ^{125}I-heparin (•---•). Opocrin eluted at a lower NaCl concentration than heparin, and was therefore less sulphated.

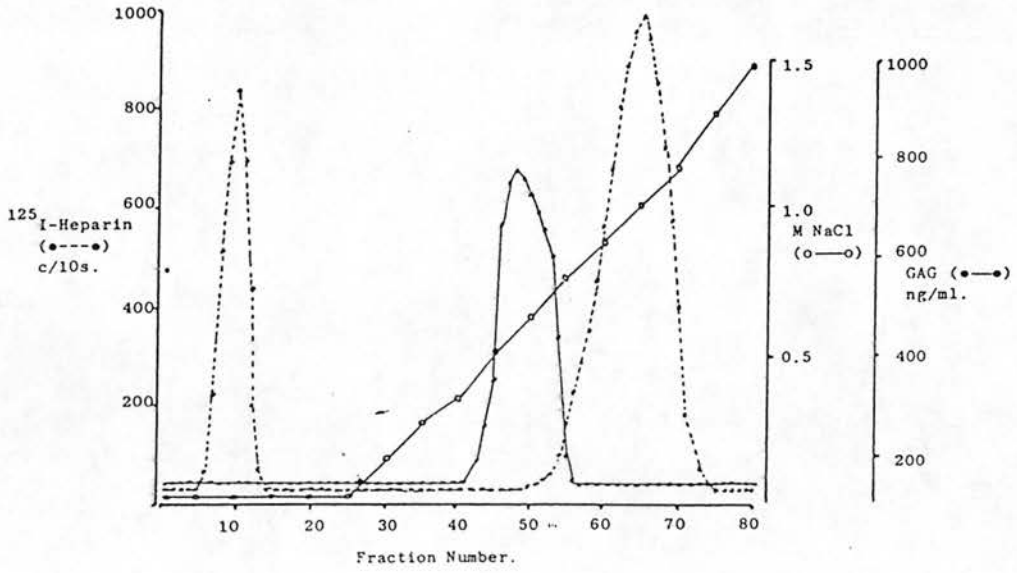


Figure 5. Elution profile from the Polybrene-Sepharose column with 0-2M NaCl gradient (o—o) of basophil-derived GAG (•—•) and ^{125}I-heparin (•---•). Cell-derived material eluted at lower NaCl concentrations than the heparin standard.

Table 3: Degree of Sulphation of Basophil-Derived GAG.

	Concentration of NaCl, where peak elution occurs.
Individual 1	0.53M - 0.73M
Individual 2	0.54M - 0.73M
Individual 3	0.52M - 0.72M

in keeping with the findings of Chapter 2:1, that GAG contained in plasma was of a similar degree of sulphation to that of heparan sulphate.

Sequential Studies.

Basophil enriched fractions of whole blood were obtained from two individuals at weekly intervals over periods of 3 and 4 weeks, to detect any changes that may occur in the GAG contained in basophils. All samples were tested for both size and degree of sulphation and the results are shown in Table 4. In general, material obtained was similar on each occasion, with the important exception of the first sample taken from one individual. On this occasion, the material obtained was identical to the heparin standard, with molecular weight of 12,000 (Figure 6a), and its elution from the Polybrene-Sepharose column was identical with the heparin standard at 1.0M NaCl (Figure 6b). When serial dilutions of fraction 17 (from Figure 6a) were assayed, the resulting curve was not parallel with the heparan sulphate standard (Figure 7a), but using the heparin assay (that is, in the presence of 50% DMSO), the material was readily assayed and produced a dilution curve parallel with the heparin standard (Figure 7b). It was concluded that on this occasion, the material released from the basophils of this individual was heparin rather than the larger, less sulphated GAG detected on all other occasions.

Effect of Pronase on Size of GAG.

Figure 8 illustrates the elution profile of the same sample as that in Figure 3 after digestion with Pronase. The material detected was reduced to a mean molecular weight of 40,000.

Table 4a: Sequential Studies on the Size of Basophil-Derived GAG.

	Molecular Weight Range of GAG.	
	Individual 1.	Individual 2.
Week 1.	12,000	2,500 - 270,000
Week 2.	< 2,000 - 220,000	< 2,000 - 500,000
Week 3.	22,000 - 250,000	2,000 - 270,000
Week 4.	25,000 - 250,000	-

Table 4b: Sequential Studies on the Degree of Sulphation of Basophil-Derived GAG.

	NaCl Conc. at which peak elution occurred.	
	Individual 1.	Individual 2.
Week 1.	0.97M - 1.03M	0.52M - 0.72M
Week 2.	0.54M - 0.73M	0.50M - 0.88M
Week 3.	0.50M - 0.73M	0.58M - 0.75M
Week 4.	0.44M - 0.75M	-

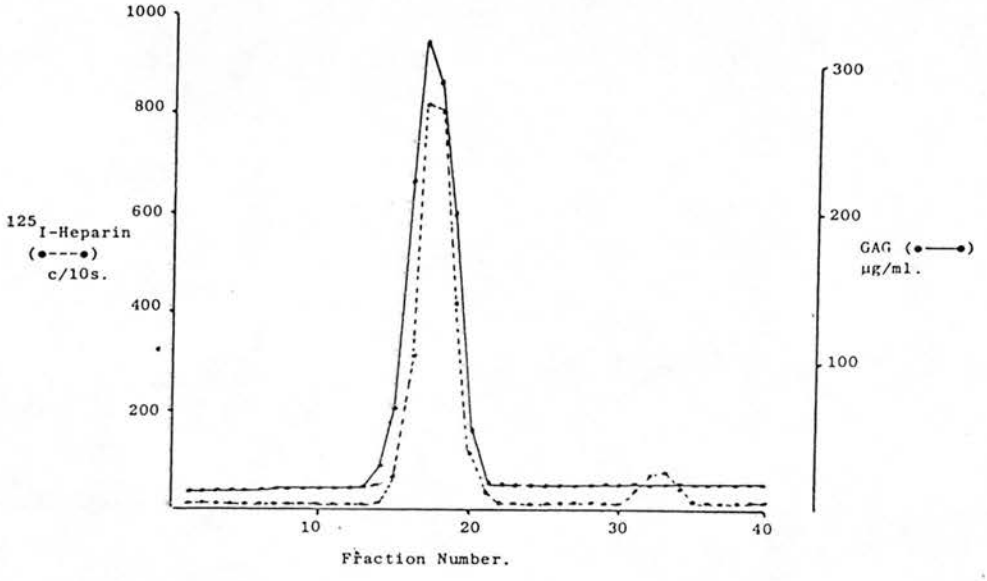


Figure 6a Elution profile from the Fractogel TSKHW-55(F) column of GAG extracted from basophils of an individual (•——•) and ^{125}I-heparin (•---•). On this occasion sample material eluted with an identical pattern to that of the heparin standard.

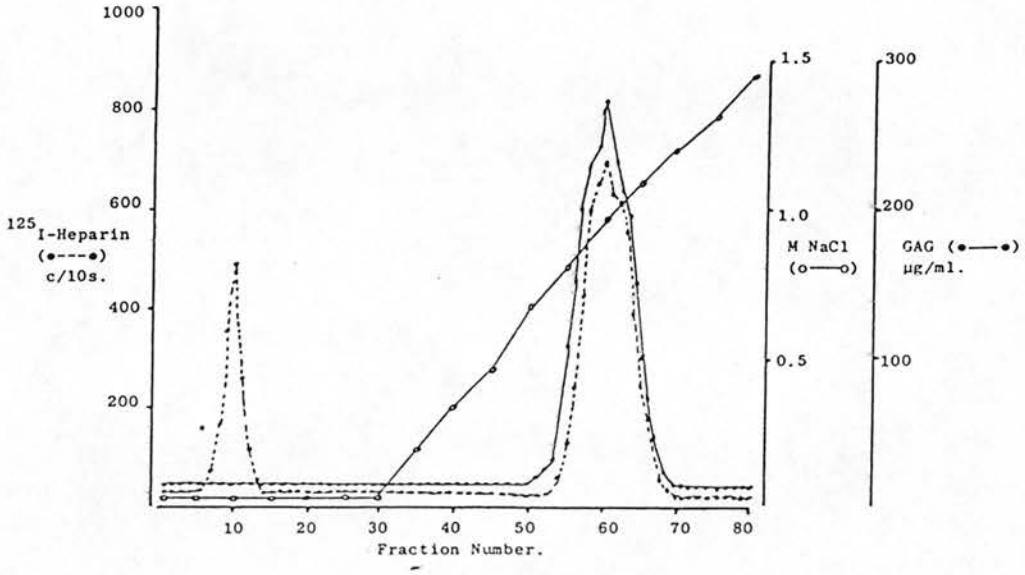


Figure 6b Elution profile from the Polybrene-Sepharose column with 0-2M NaCl gradient (o——o) of GAG extracted from basophils of an individual (•——•) and ^{125}I-heparin (•---•). On this occasion, sample material eluted at an identical NaCl concentration as the heparin standard.

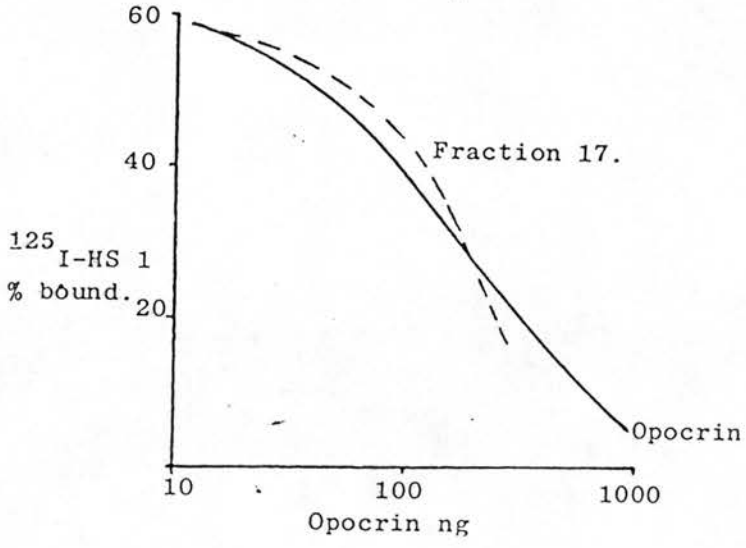


Figure 7a Serial dilutions of fraction 17 of Figure 6a were assayed and compared with heparan sulphate standard. The resulting curve was steeper than the Opocrin standard curve, indicating that the sample GAG had a higher degree of sulphation.

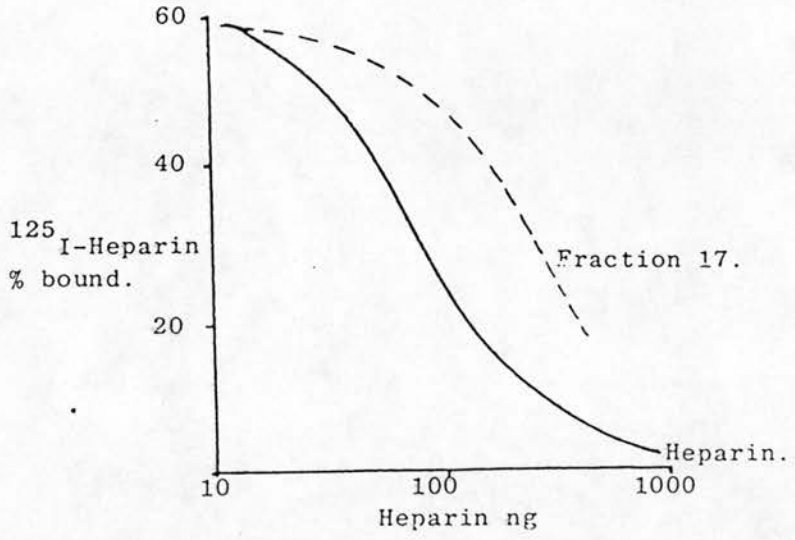


Figure 7b Serial dilutions of fraction 17 of Figure 6a were assayed in the presence of 50% DMSO, and compared with heparin standard. The resulting curves were parallel, indicating that sample material had a similar degree of sulphation to that of heparin.

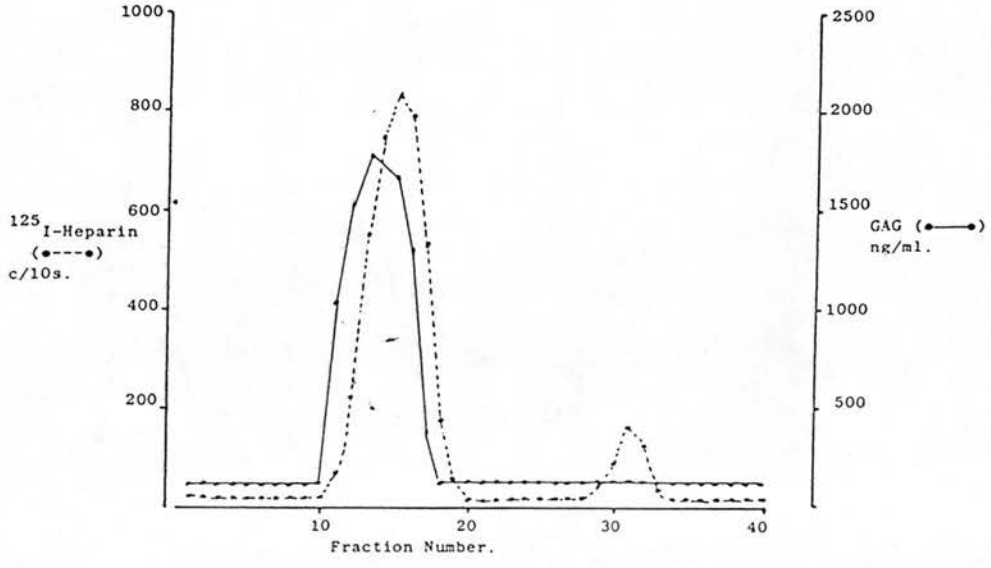


Figure 8. Elution profile from the Fractogel TSKHW-55(F) column of the same sample used for Figure 3 after digestion with Pronase (•——•) and ^{125}I-heparin (•---•). Pronase digestion reduced basophil-derived GAG to 40,000 molecular weight.

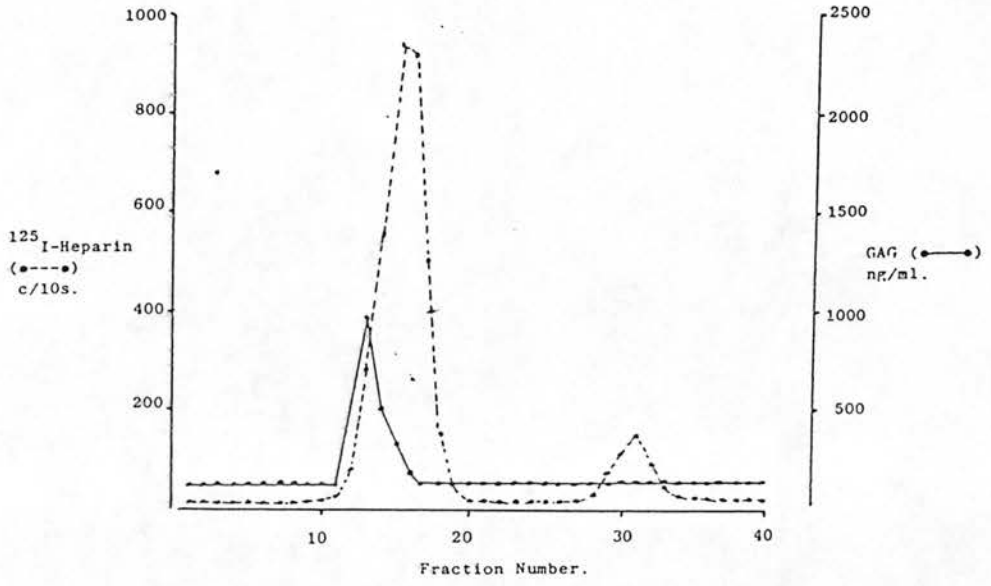


Figure 9. Elution profile from Fractogel TSKHW-55(F) column of the same sample used for Figure 4 after digestion with chondroitinase ABC (•——•), and ^{125}I-heparin (•---•). Much of the basophil-derived GAG was digested with this treatment, and undegraded material represented components of approximately 20,000 molecular weight.

Table 5: Effect of Nitrous Acid Degradation on Basophil-Derived GAG.

	GAG μ g.	
	Before treatment.	After treatment.
Per ml blood.	9.4	3.3
Per 10^6 basophils.	188.0	66.0

Effect of β -Elimination on GAG.

Size.

β -elimination of material contained in the sample (of Figure 3), caused a similar reduction in size as that of Pronase digestion; the mean molecular weight of material detected after this treatment was 40,000.

Degree of Sulphation.

After β -elimination, the basophil-derived material was eluted at 0.53-0.73M NaCl, similar to that of the untreated material of the same sample which was eluted at 0.39-0.73M NaCl. The difference in the lower limit of elution was probably due to the increased affinity of the free polysaccharide chains for Polybrene-Sepharose.

Effect of Chondroitinase ABC Digestion on Size of GAG.

Figure 9 shows the elution profile from the Fractogel TSKHW-55(F) column of the same sample as that in Figure 3 after digestion with chondroitinase ABC. The material was reduced in concentration, and the remaining undegraded material represented components of approximately 20,000 molecular weight.

(3:4). Discussion.

The design of the assay for GAG used throughout these studies is such that heparan sulphate, dermatan sulphate and oversulphated chondroitin sulphate E were all measurable at 0.2-1.0 μ g/ml, but it was insensitive to chondroitin 4-sulphate and chondroitin 6-sulphate, as described in Chapter 2:1. Although heparin could also be quantitated by this assay, it was easily distinguished from the less sulphated GAGs by the slope of the dilution curve, and by including 50% DMSO in the assay it could be made specific for

heparin. When plasma or any basophil-derived material was assayed under these conditions, no heparin was detected; heparin is therefore not a component of basophil proteoglycan. As the assay response to chondroitin 4-sulphate and chondroitin 6-sulphate was poor, the data presented in this Chapter do not conflict with previous reports of chondroitin sulphate A (chondroitin 4-sulphate) in basophilic cells (Olsson and Gardell 1967; Metcalfe et al 1984; Seldin et al 1986). It should however be emphasized that none of the more recent studies refers to normal circulating basophils and there does appear to be considerable patient variation in the type of GAGs identified (Rothenberg et al 1987). Moreover, chondroitin 4-sulphate is synthesized by a wide range of cell types, and the more highly sulphated GAGs measured by this assay are of greater physiological interest.

Human basophil-derived material contained GAGs which had molecular weights in the range of 2,300 - > 500,000 (Figure 3); high molecular weight material was probably proteoglycan in which individual GAG side chains had not yet been cleaved from the protein core. After Pronase digestion or ß -elimination, which released all single polysaccharide chains from the proteoglycan, most of the GAG had molecular weights of the order 40,000 (Figure 8). This was consistent with that of the heparan sulphate standard (Figure 2), whereas heparin side chains are usually of the order 7,000 to 25,000 (Robinson et al 1978).

Further information about the structure of basophil-derived GAG was derived from its Polybrene binding affinity and elution from the Polybrene-Sepharose column. All basophil-derived GAG eluted at a lower NaCl concentration than the heparin standard, and was therefore of a lower sulphate content (Figure 5). Moreover, the

pattern of elution of the basophil-derived material was similar to that of the heparan sulphate standard (Figure 4), indicating once more that basophil-derived GAG had a similar degree of sulphation to heparan sulphate. The GAG contained in citrated human plasma produced the same elution pattern, indistinguishable from that of the basophil-derived material, from both Fractogel TSKHW-55(F) and Polybrene-Sepharose columns; thus plasma GAG may originate from circulating basophils.

Figures 6 and 7 illustrate that during the collection of weekly sequential samples from one individual, basophil-derived GAG extracted on one occasion was heparin-like, rather than the larger, less sulphated material obtained from all other preparations (Table 4). This was undoubtedly a genuine observation, but could not be repeated, since all subsequent samples from this subject contained GAG similar to that found in all other individuals studied. These data indicate possible variability of the biochemical nature of GAGs contained in basophils in individuals; further evidence is necessary to confirm this finding.

Much of the 40,000 molecular weight GAG contained in both human plasma and normal basophilic leucocytes was degraded by digestion with chondroitinase ABC (Figure 9), and therefore had a chondroitin sulphate-like (rather than heparin-like) backbone. This material was probably the highly sulphated chondroitin sulphate E, since other chondroitin sulphates are not detectable with this assay. Some GAG resisted the chondroitinase ABC digestion (Figure 9); this resistant material was likely to be heparan sulphate, but enzyme degradation studies to test this theory were largely unsuccessful, due to the low activity of heparitinase in this experimental system. Alternative methods were therefore sought, and nitrous acid

treatment, which degrades material at N-acetyl glucosamine residues, was tested on basophil-derived GAGs.

After nitrous acid degradation, 30% of the original material remained (Table 5). Since chondroitin sulphate-like GAGs contain no N-acetyl glucosamine residues, the nitrous acid resistant basophil-derived GAG was probably chondroitin sulphate E. Thus, approximately 70% of the highly sulphated GAGs contained in human basophils, was heparan sulphate, with the remaining 30% consisting of chondroitin sulphate E. However, it must be remembered that these calculations were made from experiments using relatively crude basophil-derived GAG preparations; and as they are based on the assumption that an equivalent response of the assay to both types of GAG was obtained exact quantitation is not possible. As previously mentioned, there is also the possibility that variation of basophil-derived GAGs may occur in individuals., However, it is clear from these studies that, human basophils do contain both heparan sulphate and highly sulphated chondroitin sulphate, probably chondroitin sulphate E.

Chapter 4.

GAG in Plasma During Systemic Anaphylaxis of *Nippostrongylus brasiliensis*-primed Rats.

(1). Introduction.

The anaphylactic reactions of type 1 hypersensitivity in man are systemic or local, depending on how the shocking dose of antigen is presented in the body. The local form of anaphylaxis is likely to develop if the appropriate antigen comes into contact with respiratory or intestinal mucous membranes of a susceptible individual. Local adverse respiratory reactions may cause the symptoms of hay fever or asthma, whereas a mixed form of reaction can develop with intestinal symptoms, including urticaria and sometimes asthma. If the antigen is administered elsewhere than in the respiratory or alimentary tract, for example with drugs such as penicillin, foreign serum or perhaps an insect bite, the dramatic and rapid systemic form of anaphylaxis will develop. The symptoms include dyspnoea with bronchospasm and laryngeal oedema, urticaria, reduced blood pressure, and occasionally death.

Anaphylaxis is dependent upon the presence of IgE antibodies which have the ability to passively sensitize mast cells and basophils. Bridging two adjacent cell-bound IgE molecules by antigen leads to an allosteric membrane change which initiates a biochemical sequence leading to synthesis and release of preformed pharmacologically active mediators, detailed in Chapter 1. Anaphylactoid reactions, which represent immediate-type systemic responses in which basophils and/or mast cells are also degranulating, but IgE antibodies or other immunological mechanisms of activation are not the cause. A number of mediators are known to

induce anaphylactiod reactions, such as the carbohydrates Dextran and Mannitol (Hedin et al 1976; Lamb and Keogh 1979) and certain dyes such as bromosulphothalein (Nedwicki and Roth 1960), presumably by direct activation of basophils and/or mast cells.

A noteworthy feature of rats infected with the nematode intestinal parasite Nippostrongylus brasiliensis, has been the appearance of high titres of parasite specific and non-specific IgE antibodies in blood (Jarrett and Miller 1982). Subsequent intravenous challenge with worm antigen causes a severe adverse reaction in these animals, producing the classic symptoms of systemic anaphylaxis. The ability to simulate the events of systemic anaphylaxis in the rat has provided a useful model to study the mechanisms involved in IgE-mediated anaphylactic shock.

The IgE and mast cell/basophil system in Nippostrongylus-infected rats is thought to assist in the expulsion of the nematode from the intestine (Askenase 1980), but does render the animal susceptible to mucosal anaphylaxis. The reaction in rodents is characterized by major changes in the small intestine, including increased secretion of mucus (Urquhart et al 1965), mucosal permeability and systemic secretion of a distinctive protease enzyme, rat mast cell protease II (RMCP II), contained in the mast cells of the lamina propria (Woodbury and Miller 1982; King and Miller 1984).

Recent evidence indicates that rodents possess distinguishable populations of mast cells, termed "connective tissue mast cells" (isolated from connective tissue, lung and serosal cavities) and "mucosal mast cells" (derived from the lamina propria of the gastrointestinal tract) (Enerback 1981; Bienenstock et al 1982; Barrett and Metcalfe 1984). The major differences between the two cell types are summarized in Table 1.

Table 1: * **Two Different Types of Rodent Mast Cells.**

Mucosal Mast Cells.	**Connective Tissue Mast Cells.**
Found in mucosal membranes such as the lamina propria of the gastrointestinal tract.	Found in serosal layers, the peritoneal cavity and in solid tissues, eg. skin and tongue.
Small cells with few granules of variable shape.	Larger cells with many uniform granules.
Low, age-dependent histamine content (< 2pg/cell).	High, age-dependent histamine content (15-35pg/cell).
5-hydroxytryptamine content increased by nematode infections.	Low, age-dependent 5-hydroxytryptamine content (< 1.5pg/cell).
Contain the serine protease RMCP II.	Contain the serine protease RMCP I.
Soluble granular matrix containing unidentified low-sulphated GAG.	Less soluble granular matrix containing heparin.
Surface and cytoplasmic receptors for IgE.	Surface receptors for IgE.
Non-secretory but proliferative response to compound 48/80 and polymxin B.	Secretory response to compound 48/80 and polymyxin B.
No response to disodium cromoglycate and theophylline.	Activation inhibited by disodium cromoglycate and theophylline.
Proliferative response to nematode infections.	Response to nematode infections unclear.
Short life span, half life approx. 40 days.	Very long life span.
Thymus-dependency of proliferation.	Thymus-independency of age-dependent proliferation and growth.
Main repository of histamine in the gut mucosa.	Main repository of histamine in peritoneum and tongue.

* Summarized from Enerback 1981; Wingren *et al.* 1983; Seldin *et al* 1985; Gleich *et al* 1986.

There are two major hallmarks which distinguish the mucosal and connective tissue cell that are of interest to the studies of this Chapter:

a. Protease content.

The mucosal mast cells of rodents contain the highly soluble neutral serine protease RMCP II, of M_r approximately 25,000 (Woodbury et al 1978a; Woodbury and Neurath 1978; Woodbury and Miller 1982; Woodbury et al 1984), whereas connective tissue mast cells contain a M_r 28,000 protease, typed with monospecific antisera as RMCP I (Woodbury et al 1978b). The exclusive cellular localization of RMCP II has been exploited, and used as a marker to study the involvement of mucosal mast cells in the parasitised gut of the rat, and to monitor its functional activity during systemic anaphylaxis (Miller et al 1986).

b. Glycosaminoglycan content.

The predominant GAG contained in the granules of connective tissue mast cells is heparin (Enerback et al 1974; Enerback et al 1976), whereas mucosal mast cells contain a GAG with a lower degree of sulphation (Stevens and Austen 1981; Enerback et al 1985) the exact nature of which has yet to be determined. However, similarities have been reported between rat mucosal mast cells and mast cells derived from the bone marrow of mice, which contain chondroitin sulphate E (Razin et al 1981; Razin et al 1982).

Homogeneous populations of mast cells resembling the mucosal mast cell subclass have been obtained in vitro from rat bone marrow cultured in the presence of conditioned medium from antigen-activated immune mesenteric lymph nodes (Haig et al 1982;

Haig et al 1983). These mast cells stain with alcian blue but not safranin, they contain RMCP II but no RMCP I (McMenamin et al 1987), have a low histamine content, and proliferate under T cell regulation. Their predominant proteoglycan, which is not heparin, has a relatively low degree of sulphation (Haig et al 1984), but its exact nature remains open to question. These cells have been used for isolation and characterization of mucosal mast cell-associated GAG.

Primary infection of rats with N. brasiliensis initiates a series of changes and events at a cellular level (Miller et al 1986), and among the most noteworthy features is the exponential increase of mucosal mast cells (Miller and Jarrett 1971). Following intravenous challenge with homologous worm antigen, extensive mucosal damage occurs in primed rats, with depletion of mucosal mast cells from jejunal mucosa, accompanied by the systemic release and decreased mucosal concentration of RMCP II (Miller et al 1983; King and Miller 1984).

Since GAG is localized in the same cytoplasmic granules of mature mast cells as is RMCP II, this Chapter describes an investigation of some of the properties of mucosal mast cell-derived GAG during systemic anaphylaxis, and highlights this as yet another example of the biochemical and functional differences between rodent mucosal and connective tissue mast cells.

(4:2). Materials and Methods.

Materials.

Reagents.

'Thrombotect' tubes were purchased from Abbott Laboratories Ltd, Wokingham, Berkshire, and alkaline phosphatase-conjugated goat anti-rabbit IgG from Sigma Chemicals Ltd, Poole, Dorset. Plastic, 96-well microtiter plates (Titertek), and rat basophil leukaemia cells (RBL-1, ATTC No. CRL 1378) were purchased from Flow Laboratories, Rickmansworth, Herts.

Methods.

This Chapter describes a collaborative study with Dr HAF Miller and Dr SJ King, who performed all experiments involving the infection and challenge of rats with worm antigen. They also performed all RMCP II measurements, histology, cell counts, and cultured the rat bone marrow cell preparations. The methods used were as follows:

Animals.

19 female outbred Wistar rats were used, 15 of which were immunised 3 and 8 weeks prior to each experiment, by subcutaneous injection with 6,000 N. brasiliensis larvae. The techniques of parasite culture, recovery and ennumeration are those described by Nawa and Miller (1978), and Miller et al (1981). The preparation of whole worm antigen was as described by Nawa et al (1981), and it was administered intravenously to groups of fasted (24h) rats in the following doses:

Group.	No of Animals	Dose of Worm Antigen
1.	4	Immune rats challenged with 1000 worm equivalents (w.e.).
2.	3	Immune rats challenged with 500 w.e.
3.	4	Immune rats challenged with 250 w.e.
4.	4	Immune rats challenged with saline only.
5.	4	Control naive rats challenged with 500 w.e.

Animals were anaesthetised 60min after intravenous challenge, and bled from the carotid artery.

Preparation of Samples.

Serum.

Blood was collected, allowed to clot, and serum removed after centrifugation. Sera were stored at -20°C before assay for RMCP II.

Plasma.

Blood was collected into the anticoagulant 'Thrombotect' (Abbott), the plasma separated by centrifugation and stored at -70°C before assay for GAG.

Tissues.

The techniques used were those described by Woodbury and Miller (1982), which involved the removal of the small intestines of each animal. The intestines were divided into two equal lengths and each portion perfused with 10ml of saline plus 10ml of air (Nawa 1979). Gut perfusates were then stored at -20°C.

A 30cm length of jejunum from 15cm behind the pylorus of each rat was opened and washed three times in 100ml of phosphate buffered saline (PBS) at 4°C. The gut segments were washed twice in 0.15M KCl, blotted to remove exess fluid, and finally weighed. The

tissues were then minced with scissors in 10ml 0.15M KCl at 4°C and homogenized three times for 1 min intervals in a teflon/glass homogenizer cooled with crushed ice. After centrifugation (300g; 4°C), the homogenate supernatant was removed and clarified by ultracentrifugation (36,000g; 30min; 4°C) using a Beckman L2 65B ultracentrifuge. The samples were then freeze-dried and stored at 4°C.

Measurement of RMCP II.

Serum.

The measurement of RMCP II in rat serum was by a competitive enzyme-linked immunosorbent assay (ELISA) developed by Miller et al (1983). Basically this involved the incubation of unknown samples and standards with rabbit anti-RMCP II, in plastic 96-well microtitre plates coated with RMCP II. After incubation (37°C; 1-2h), the wells were washed three times with a solution of PBS/1% BSA/0.05% Tween 20, before incubation for 1h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, Poole, Dorset). After a further three washes with PBS/BSA/Tween 20, the wells were incubated with the substrate Na-p-nitro-phenyl phosphate for 20-30min. The plates were finally read in a Titertek Multiscan Reader (Titertek, Flow Laboratories), and results expressed as ng RMCP II/ml of serum.

Tissues.

Levels of RMCP II were measured by radial immunodiffusion as described by Woodbury and Miller (1982). Samples were added to radial immunodiffusion plates prepared with 1% agar (as previously described by Woodbury and Neurath 1978), containing rabbit anti-RMCP II antibody. The precipitin bands were allowed to develop in a moist atmosphere at room temperature for 24 hours, when the

diameters of the bands were measured, and the results expressed as μ g RMCP II/g wet weight of tissue (Woodbury and Miller 1982).

Histology and Cell Counts.

Samples of jejunum were collected and placed in either Carnoy's fluid or 4% paraformaldehyde. Tissues were embedded in wax, and sections were stained either for proteoglycan using Toluidine blue, pH 0.5 (Enerback 1966), or with Naphthol AS-D chloroacetate to demonstrate serine esterases (Newlands et al 1984).

Measurement of GAG.

Samples of plasma, gut perfusates and gut homogenates were pretreated and assayed for GAG according to the method described in detail in Chapter 2:1.

Time Course of Both GAG and RMCP II Release following Systemic Anaphylaxis.

A total of 43 female Wistar rats (200-250g) were used and allocated randomly to the following groups:

Group.	No.	Treatment.
1.	5	Immune rats bled 5min after antigen challenge.
2.	10	Immune rats bled 15min after antigen challenge.
3.	9	Immune rats bled 30min after antigen challenge.
4a.	10	Immune control challenged with saline only.
4b.	4	Naive control challenged with saline only.
4c.	5	Naive control challenged with antigen.

Immune rats were bled 5min (Group 1), 15min (Group 2) or 30min (Group 3) after challenge with 500 w.e., whereas control animals in Group 4 were bled 30min after challenge with saline or antigen at 500 w.e.

Samples of plasma and serum were obtained from each animal and examined for the presence of GAG and RMCP II.

Release of Histamine into Plasma following Systemic Anaphylaxis.

24 female outbred Wistar rats of 200-250g were used and randomly allocated to groups of 4 as follows:

Group	Dose of Worm Antigen (w.e.).
1.	Immune rats challenged with 1000 w.e.
2.	Immune rats challenged with 500 w.e.
3.	Immune rats challenged with saline only.
4.	Naive control rats challenged with 1000 w.e.
5.	Naive control rats challenged with 500 w.e.
6.	Naive control rats challenged with saline only.

The immune rats were challenged 7 days after secondary infection with 4000 N. brasiliensis, and were bled out under anaesthetic 30min after antigen challenge. Plasma and gut tissue samples were obtained from each animal and examined for the presence of GAG, histamine and RMCP II.

Measurement of Histamine.

Samples of plasma were pretreated and assayed for GAG according to the method of Shore et al (1959), described in detail in Chapter 2:2.

Characterization of GAG.

Cultured Rat Bone Marrow Derived Cells.

Rat bone marrow cells cultured in Iscove's medium with 10% horse serum, and 30% conditioned medium (Haig et al 1982) were counted and centrifuged (150g; 15min; 4°C). The supernatant was removed, and cells (6×10^6) washed once in 0.01M phosphate buffer with 0.9% NaCl.

Centrifugation was then repeated, supernatant removed, and the cell pellet was resuspended to 2ml using 1M NaCl containing:

0.05M sodium acetate,

0.01M EDTA,

0.1M 6-aminohexanoic acid,

0.005M benzamidine HCl.

All subsequent steps to extract GAG from cells, and determination of its size and degree of sulphation are as described in Chapter 3. All steps were performed as near to 4°C as possible using an ice bath, and supernatant fluids retained and assayed for GAG.

Cultured Rat Basophil Leukaemia Cells.

Rat basophil leukaemia cells (Flow Labs Ltd.) cultured in minimum essential medium with Earle's salts and 10% foetal calf serum, were counted and treated as rat bone marrow cells above, to extract and characterize the GAG.

Rat Plasma.

Plasma samples obtained from 4 immune rats challenged with 1000 w.e. were pooled, and assayed to determine GAG content. This material was then prepared for the Fractogel TSKHW-55(F) column as described in Chapter 3, to determine the molecular weight range of GAGs released into rat plasma following systemic anaphylaxis.

Treatment of plasma with chondroitinase ABC is also described in Chapter 3.

(4:3). Results.

Release of Both GAG and RMCP II from Mucosal Mast Cells following Systemic Anaphylaxis in the Rat.

The contents of GAG and RMCP II in the samples studied are summarized in Table 1.

Group	GAGµg/ml.			RMCP IIµg/ml.		
	Plasma	IP	GH	Serum	IP	GH
1.	14.6±3.1	21.1±3.6	21.4±6.8	770±129.4	1471.5±547	1020 ± 180
2.	15.8±2.3	24.7±3.6	17.6±2.8	670±216.3	2085 ± 663.8	1220 ± 121 2
3.	14.2±1.8	21.2±1.4	12.3±4.6	635±102.1	1144.5±316.9	1425 ± 172.3
4.	1.4±0.3	7.5±8.3	15.1±1.6	0	0	2452.5±357.8
5.	1.7±0.7	17.4±5.0	15.3±3.5	0	0	720 ± 0

Table 1: **Release of GAG and RMCP II from mucosal mast cells following systemic anaphylaxis.**

IP= Intestinal perfusates.

GH= Gut homogenates.

Mean values are shown with standard deviation.

RMCP II was present only in the sera of immune rats challenged with worm antigen (groups 1-3), and in these same groups there was a 10-fold increase in plasma GAG concentration, compared with that of animals in groups 4 and 5. Figure 1 shows that the patterns of release of GAG and RMCP II were similar, and neither was dose dependent. Nevertheless, there was a highly significant correlation between the concentrations of RMCP II released into serum, and GAG released into plasma ($y=2,110 + 17x$, $r=0.93$, $p< 0.001$), which may suggest that they have a common origin. This is illustrated in Figure 2.

RMCP II was present in the intestinal perfusates of animals only in groups 1-3, in which there was a concomitant, highly significant depletion of RMCP II from samples of gut tissue, and a decrease in the numbers of mast cells in the intestinal mucosa (Figure 3).

The redistribution of GAG in the gut during systemic anaphylaxis did not appear to follow that of RMCP II (Figure 4), since GAG was present in the gut perfusates and gut homogenates of animals in all 5 groups (Table 1). There was considerable release of GAG into gut perfusate of naive controls after antigen challenge (17.42, SD=5.0 μ g/ml perfusate), and accurate measurement of mucosal GAG was restricted, due to interference and high background levels of tissue GAG in these samples.

To further examine the theory that proteoglycan and serine esterases were depleted at the same time from discharging mucosal mast cells, two methods were used to stain these cells:

a. Toluidine blue, which detects mucopolysaccharides contained in tissues,

and **b.** naphthol AS-D chloroacetate which demonstrates the presence of specific esterases in tissue sections.

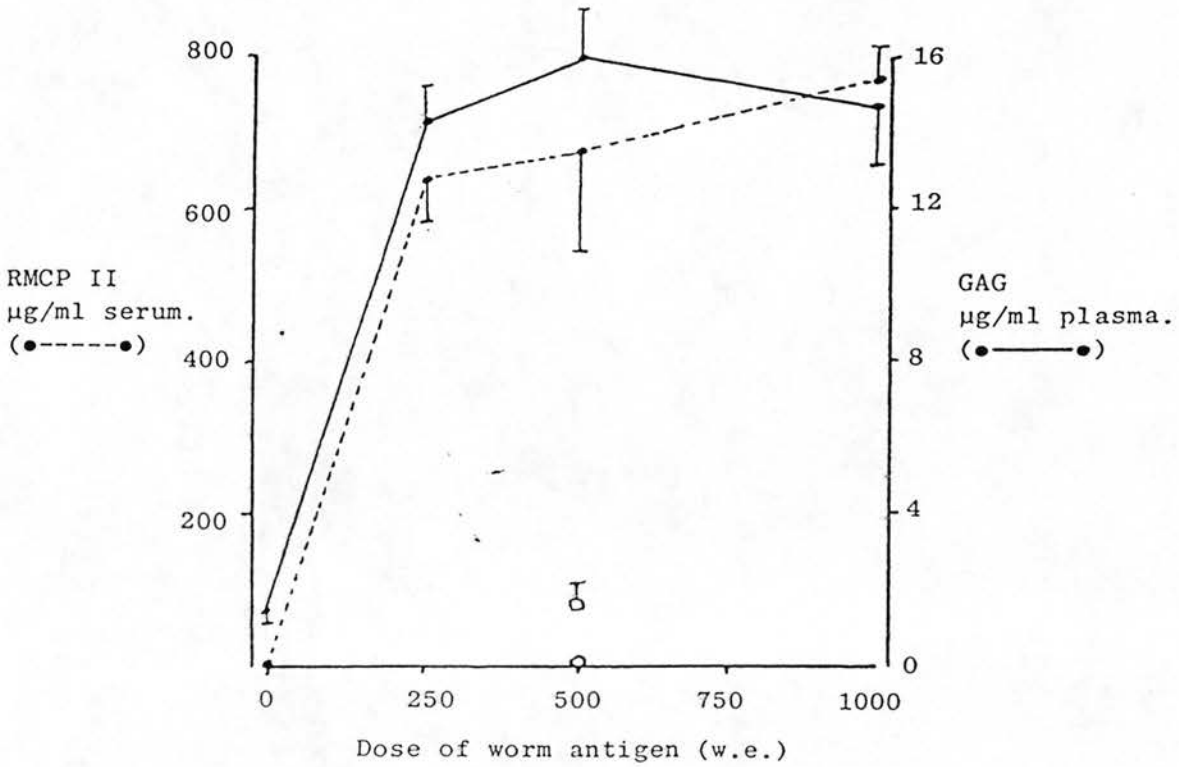


Figure 1: The contents of GAG in plasma (●——●) and RMCP II (●---●) in serum (mean ±SEM), of immune rats are shown against the intravenous dose of worm antigen. Naive control rats challenged with 500 w.e. of antigen released some GAG(□), but not RMCP II (o).

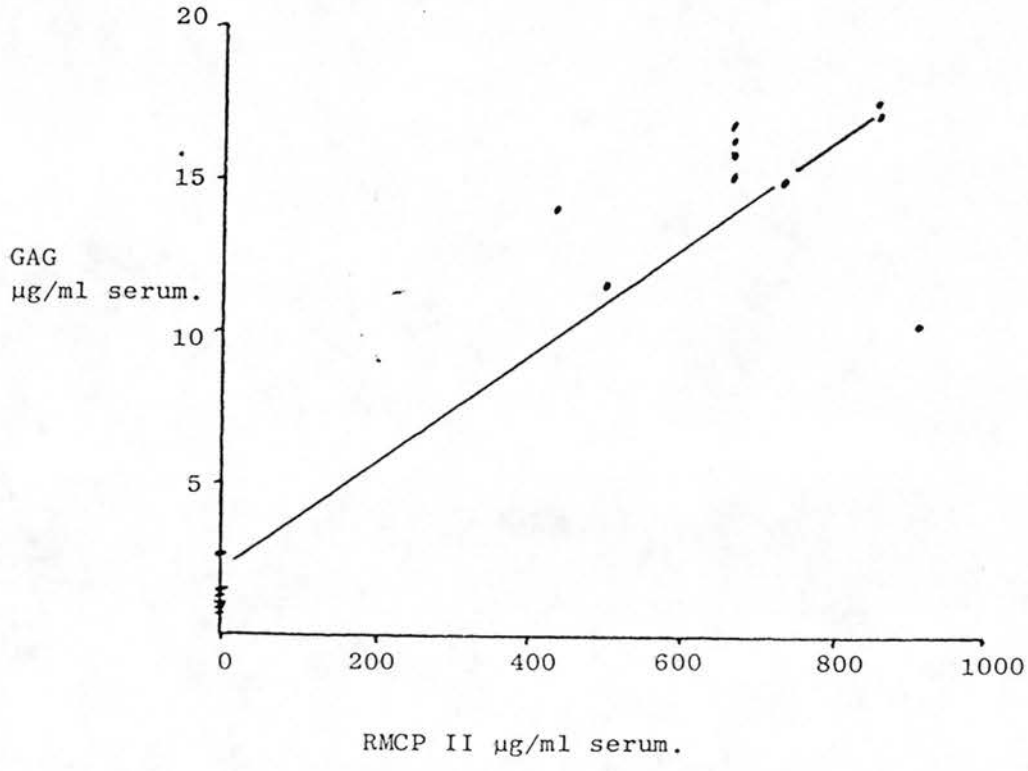


Figure 2. Regression analysis of the concentrations of RMCP II in serum against GAG in plasma ($y = 2{,}110 + 17x$, $r = 0.93$, $p < 0.001$). Plasma was not obtained from one rat given 500 w.e.

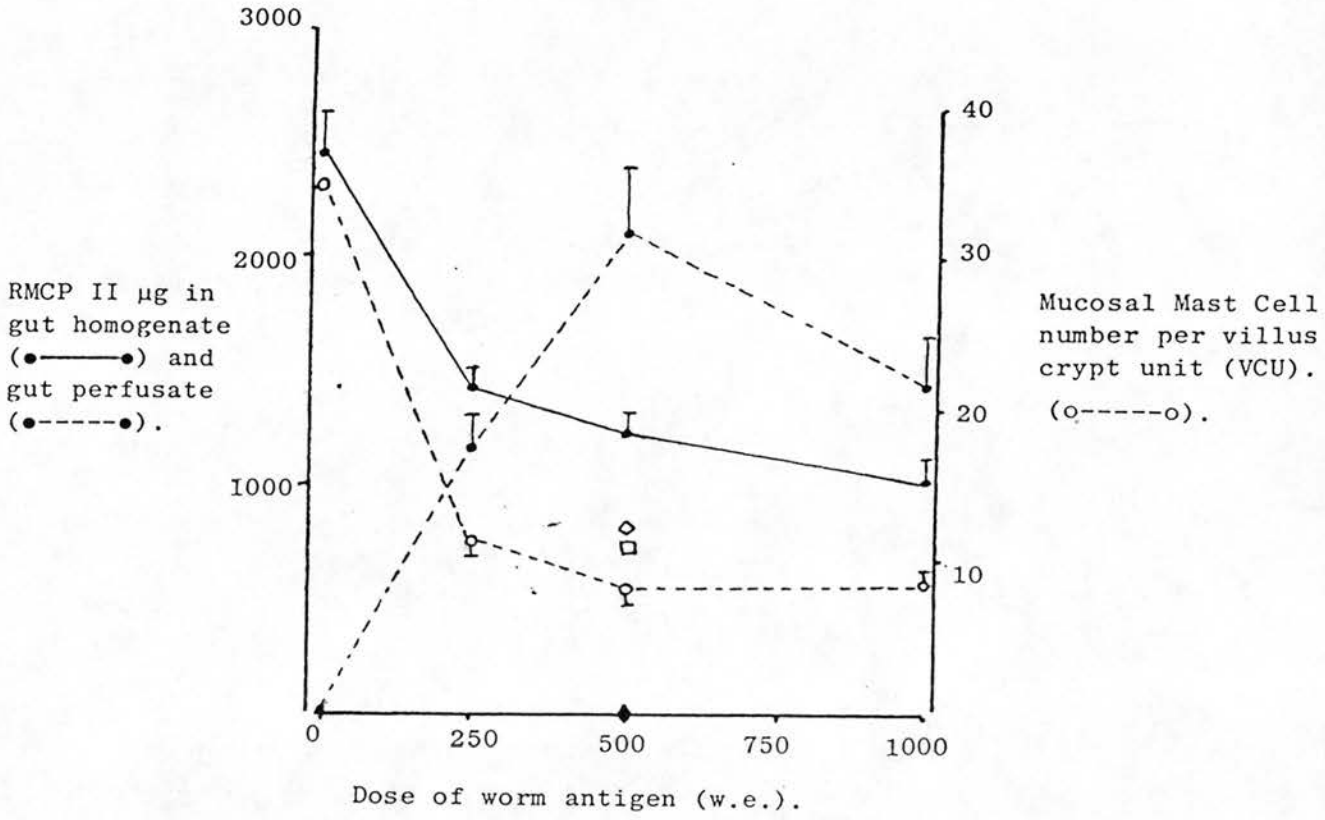


Figure 3. The concentrations of RMCP II (mean± SEM) in intestinal perfusates (●---●) and jejunal mucosa (●——●) are shown against the dose of worm antigen, with the number of mucosal mast cells (MMC) per villus crypt unit (VCU) detected with Toluidine blue (mean ± SEM). RMCP II concentrations in perfusates (♦) and homogenates (□), and the number of MMC per VCU in naive control rats challenged with 500 w.e. of antigen are also indicated.

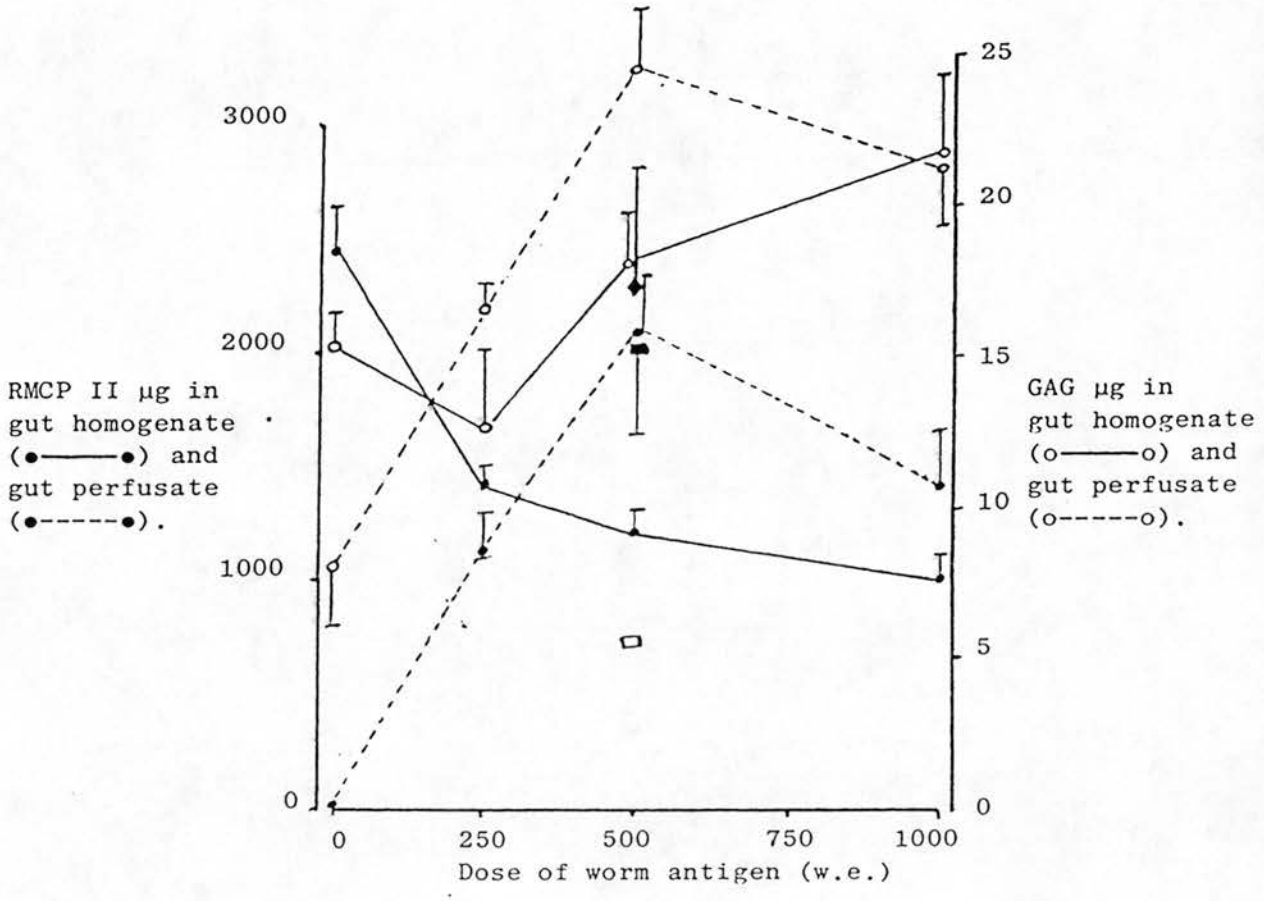


Figure 4. The concentrations of GAG in gut homogenate (o——o) and gut perfusate (o---o), and RMCP II in gut homogenate (●——●) and gut perfusate (●---●) (mean ± SEM), are plotted against the dose of worm antigen. GAG was present in both gut perfusate (♦) and gut homogenate (■) of naive control rats challenged with 500 w.e., but RMCP II was present only in gut homogenate (□).

Staining with both naphthol AS-D chloroacetate and Toluidine blue indicated mast cell depletion from the mucosa (Figure 5). There was a highly significant relationship between the numbers of mast cells detected with these 2 methods ($y=-2.3 + 1.1x$, $r=0.98$, $p< 0.001$) (Figure 5), suggesting a common origin for proteoglycan and RMCP II release.

Time Course of GAG and RMCP II Release following Systemic Anaphylaxis.

The content of GAG and RMCP II in the samples studied are summarized in Table 2.

The release of GAG into plasma and RMCP II into the sera of immune rats occurred with different time courses (Figure 6). The levels of RMCP II released into serum increased steadily, approaching a plateau 30min after worm antigen challenge; GAG was released more slowly (Figure 6). Neither GAG nor RMCP II was completely released by 30min after antigen challenge, and clearly a longer experimental period would have been required to allow for total release. There was no RMCP II, and very little GAG (< 0.15ug/ml), detected in the sera of naive rats given saline or antigen 30min after challenge.

Release of Histamine into Plasma following Systemic Anaphylaxis.

The content of histamine, GAG and RMCP II in the samples studied are summarized in Table 3.

Immune rats challenged with antigen (groups 1 and 2) were found to have increased levels of histamine as well as GAG and RMCP II in plasma (Figure 7a), when compared with control groups challenged with saline only (group 3), or naive controls (groups 4-6), (Figure 7b).

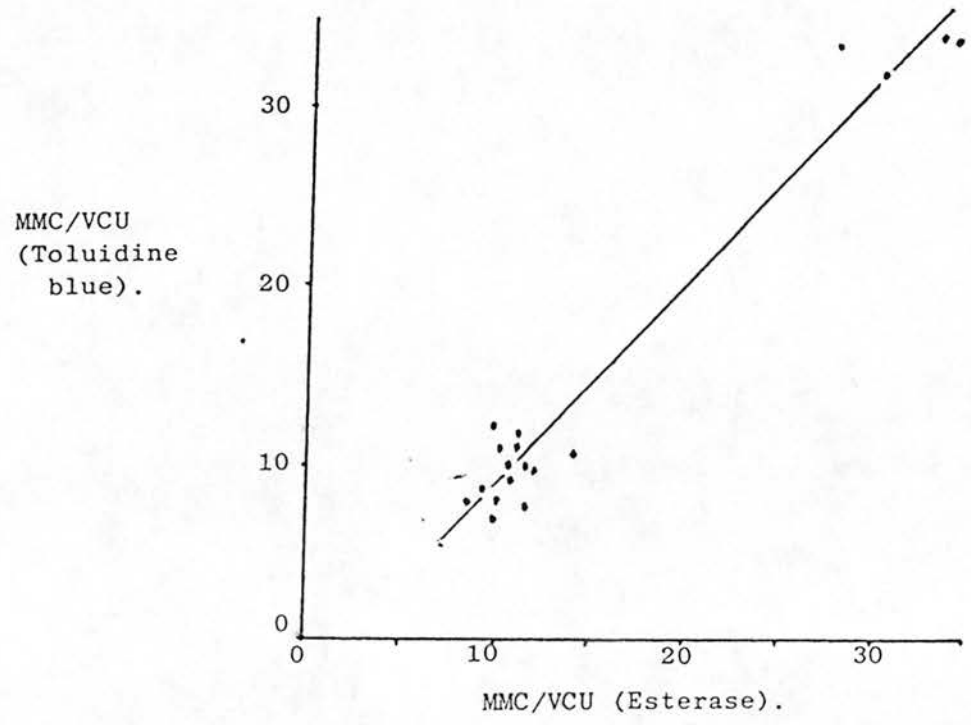


Figure 5. Regression analysis demonstrating a highly significant correlation between the numbers of MMC enumerated after staining with Toluidine blue and with naphthol AS-D chloroacetate ($y = -2.3 + 1.1x$, $r = 0.98$, $p < 0.001$).

Group.	GAG in Plasma µg/ml.	RMCP II in Serum µg/ml.
1.	0.94 ± 0.32	207 ± 93.0
2.	84.1 ± 53.5	375.5 ± 139.8
3.	281.85 ±127.4	439.4 ± 70.1
4a.	0.28 ± 0.14	0
4b.	0,06 ± 0.03	0
4c.	0.14 ± 0.04	0

Table 2.: **Time course of GAG and RMCP II release following systemic anaphylaxis.**

Mean values are shown with standard deviation.

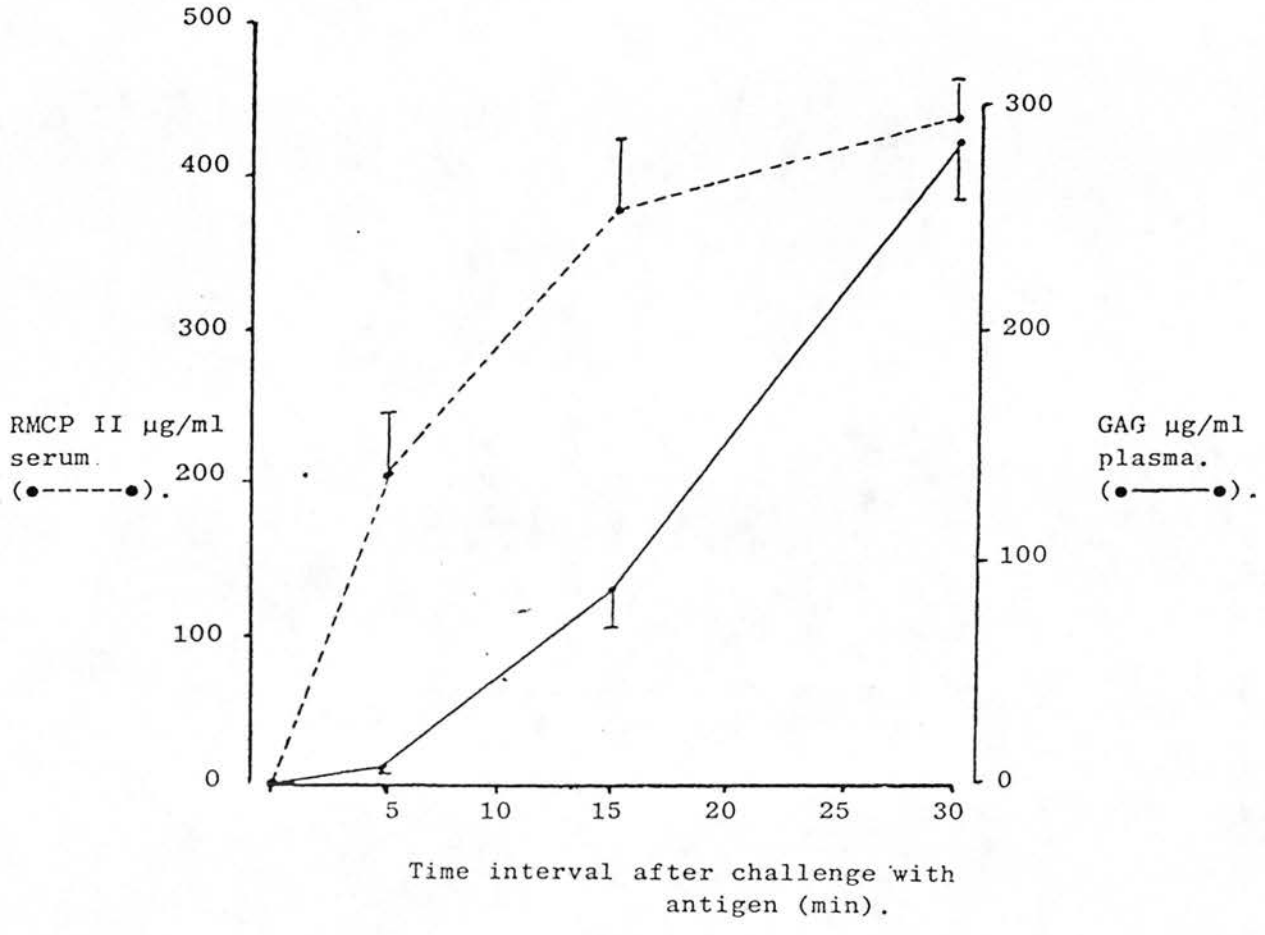


Figure 6. The contents of GAG in plasma (●——●) and RMCP II in serum (●---●) (mean ± SEM) are plotted against time interval after challenge with 500 w.e. of worm antigen. Naive rats challenged with 500 w.e. did not have detectable levels of serum RMCP II, 30min after challenge with either saline or 500 w.e., and plasma GAG levels were low at < 0.15ug/ml.

Group.	GAG in plasma µg/ml	Histamine in plasma ng/ml	RMCP II in plasma µg/ml	RMCP II in Gut tissue µg/ml
1.	89.2 ± 87.5	153.5 ± 75.1	520.0 ± 88.8	2790.0 ± 420.0
2.	33.1 ± 34.4	65.2 ± 47.1	392.5 ± 205.0	3300.0 ± 569.2
3.	0.5 ± 0.1	35.6 ± 7.0	0	5000.0 ± 1191.5
4.	0.4 ± 0.1	33.4 ± 6.6	0	1578.8 ± 151.2
5.	0.5 ± 0.1	30.2 ± 3.2	0	1620.0 ± 120.6
6.	0.65 ± 0.3	29.9 ± 3.2	0	1631.2 ± 165.8

Table 3.: **Release of histamine from mucosal mast cells following systemic anaphylaxis.**

Mean values are shown with standard deviation .

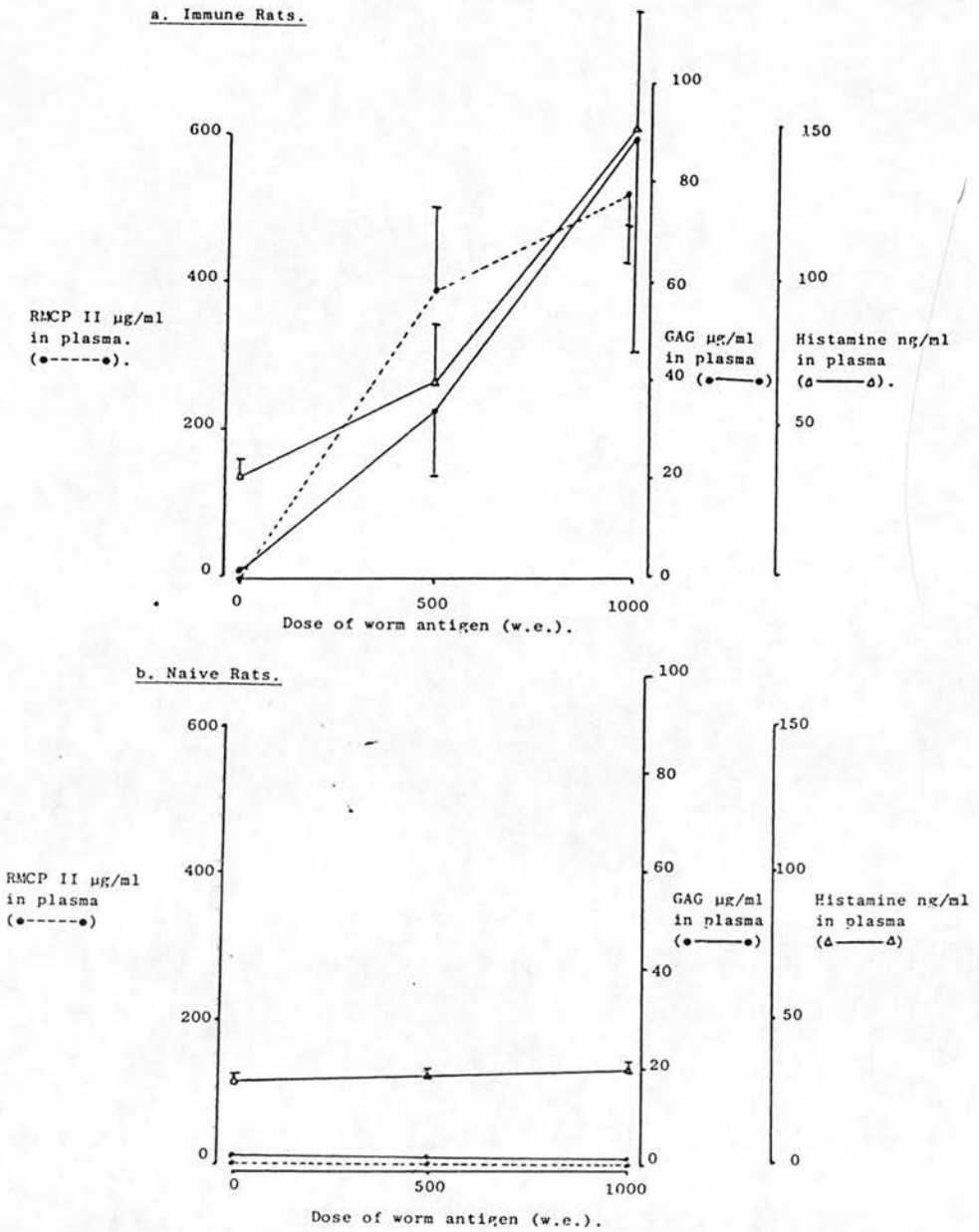


Figure 7. The plasma concentrations of GAG (•——•), RMCP II (•---•) and histamine (Δ---Δ) (mean ± SEM) are plotted against the dose of worm antigen for **a.** immune rats, and **b.** naive rats.

The release of these substances into plasma (Figure 7a), and depletion of RMCP II from gut tissue (Figure 8), increased with the dose of antigen administered.

Since histamine release into plasma followed the same pattern as GAG and RMCP II following systemic anaphylaxis, a common origin for all 3 was likely, the mucosal mast cell being the most obvious source.

Characterization of GAG.

Cultured rat bone marrow derived cells.

The GAG content of supernatant fluids after centrifugation was low, not exceeding 435 ng/ml.

a. Molecular Weight of GAG.

The elution profile obtained from the Fractogel TSKHW-55(F) column illustrates the molecular size distribution of GAG in the sample, compared with the internal marker, ^{125}I-heparin. Figure 9 shows two elution profiles of two separate preparations of these cells, and on each occasion most of the material released was of similar size. Figure 9a had a small peak representing material of $\geq$ 500,000 molecular weight, but the major GAG peak occurred at 40,000. This was repeated, (Figure 9b) and a single peak of 40,000 molecular weight was obtained. The smaller, second peak of radioactivity detected in later fractions was unbound ^{125}I.

b. Degree of sulphation of GAG.

The elution profile from the Polybrene column (Figure 10) shows that GAG released from these cells is less sulphated than the ^{125}I-heparin marker. Two small peaks were present in fractions eluted at low salt concentrations, but most of the GAG was

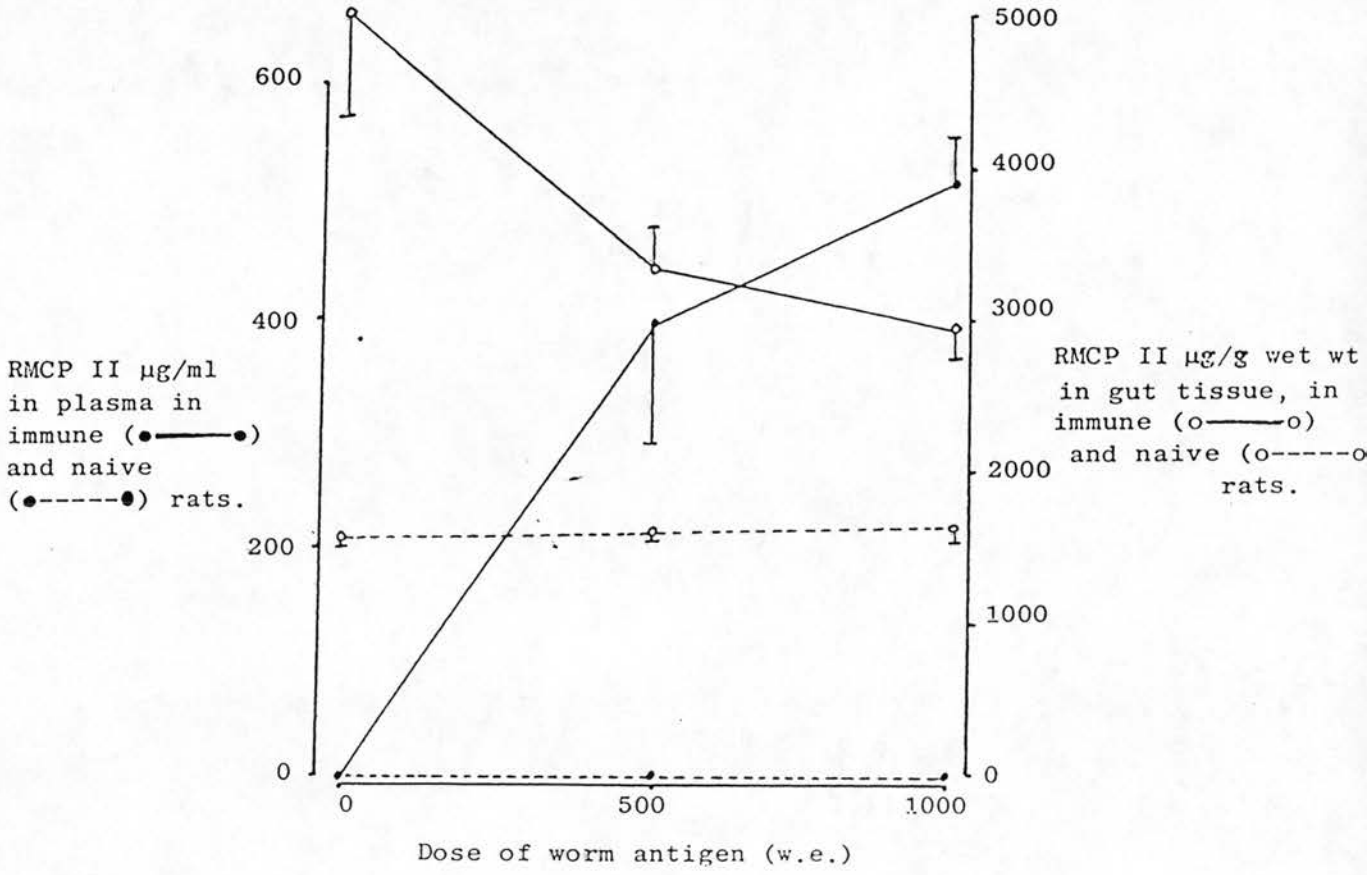


Figure 8. The concentrations of RMCP II in plasma of immune (●——●) and naive (●---●) rats, and the concentrations of RMCP II in the jejunal mucosa of immune (o——o) and naive (o---o) rats, are plotted against the dose of worm antigen.

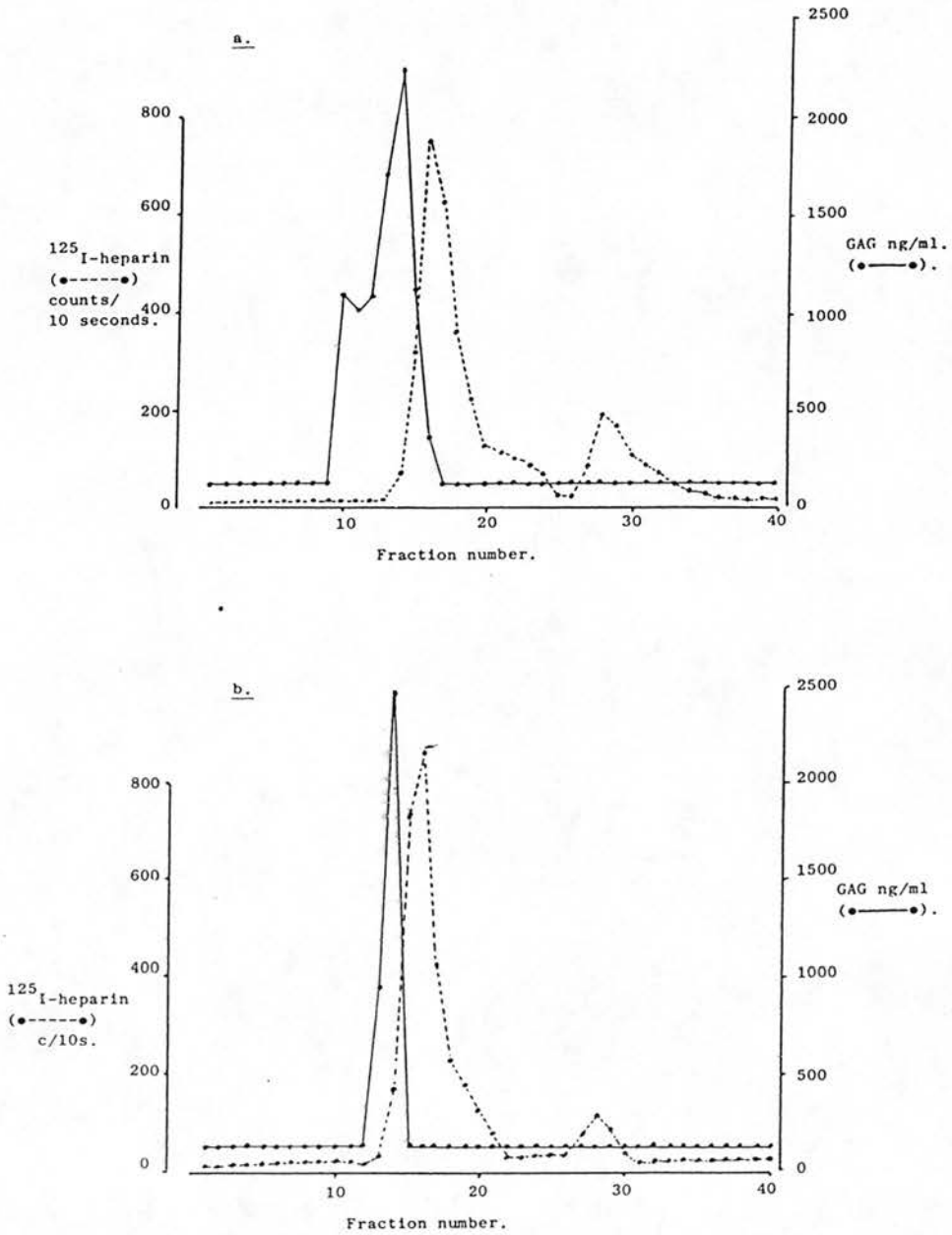


Figure 9. Two elution profiles from Fractogel TSKHW-55(F), of GAG extracted from cultured rat bone marrow derived mast cells on separate occasions. Similar patterns were obtained, with most of the material eluting at a position equivalent to 40,000 molecular weight. (•——• = sample material; •---• = ^{125}I-heparin tracer).

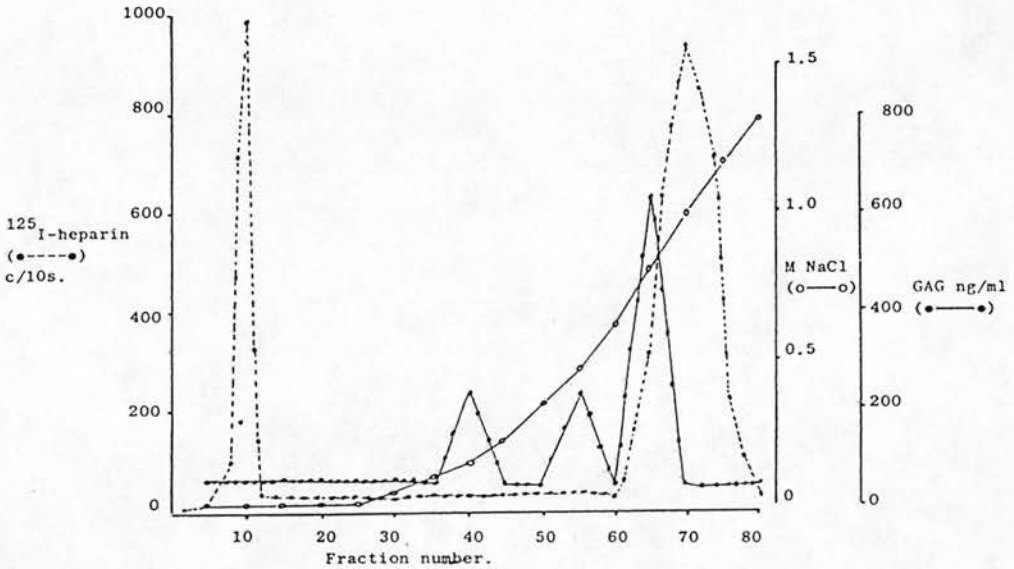


Figure 10. An elution profile from Polybrene-Sepharose with 0-2M NaCl gradient (o——o) of GAG extracted from rat bone marrow derived mast cells (•——•), and ^{125}I-heparin (•---•). Cell derived material eluted at lower NaCl concentrations than heparin, and was therefore less sulphated.

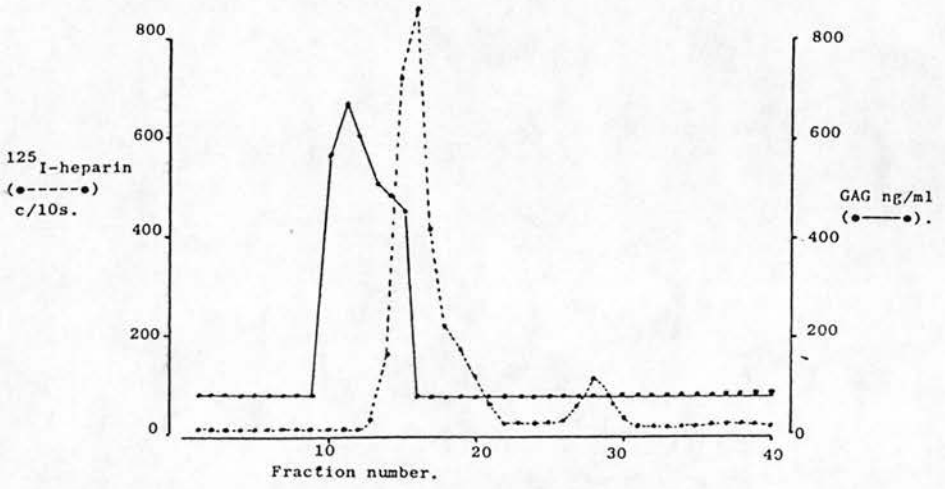


Figure 11. Elution profile from Fractogel TSKHW-55 (F) of GAG extracted from rat basophilic leukaemia cells (•——•) and ^{125}I-heparin tracer. The material eluted had a wide range of high molecular weight GAGs from 20,000 to $\gg$ 500,000.

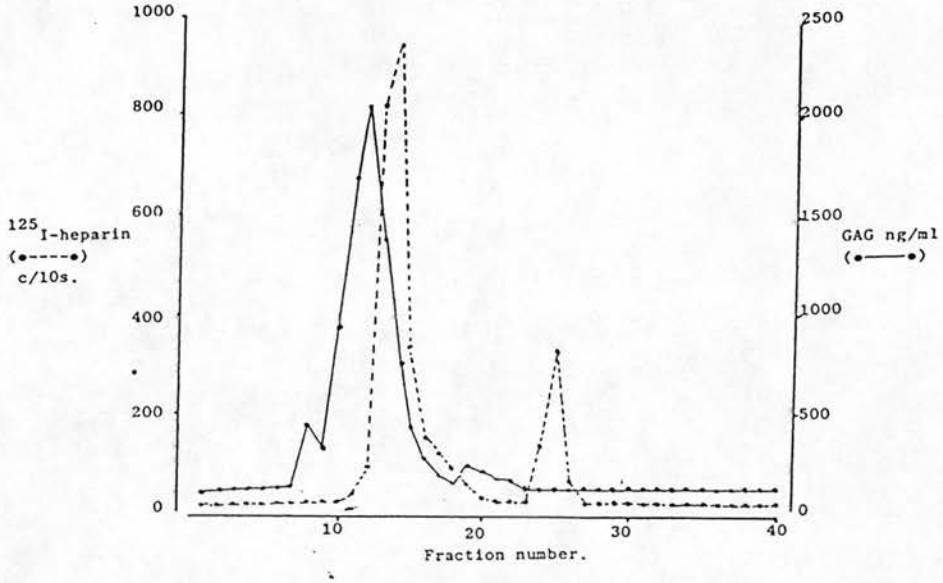


Figure 12. Gel filtration of plasma obtained from immune rats challenged with 1000 w.e. (•——•) and ^{125}I-heparin tracer (•---•) on Fractogel TSKHW-55(F). Most of the plasma-derived material was of 40,000 molecular weight.

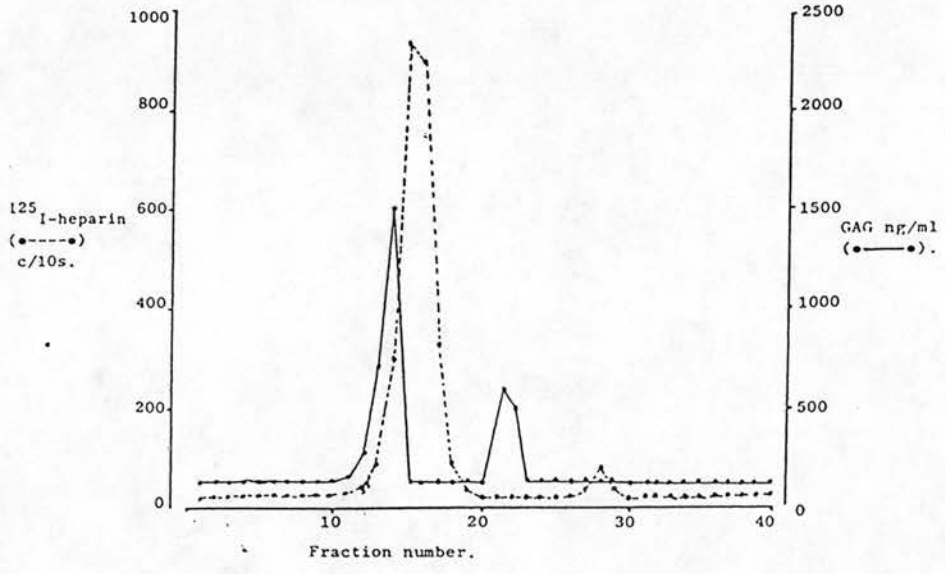


Figure 13. Plasma obtained from immune rats challenged with 1000 w.e. was treated with chondroitinase ABC (• •), and gel filtered on Fractogel TSKHW-55(F), with ^{125}I-heparin tracer (•---•). Most of the plasma GAG was digested by chondroitinase ABC, leaving a residual peak of molecular weight 22,000.

displaced from the column when the concentration gradient reached 0.8M NaCl. The unbound radioactivity detected in fractions 4-12 was free ^{125}I.

Cultured rat basophil leukaemia cells.

A wide range of high molecular weight GAG, which was not heparin, was released from these cells (Figure 11). Material representing intact proteoglycan of $\geq$ 500,000 molecular weight, as well as individual side chains of at least 20,000 molecular weight, were detected.

Rat plasma.

Of the GAGs released into plasma during systemic anaphylaxis, the majority had a molecular weight of 40,000 (Figure 12), similar to that released from the cultured bone marrow cells. There were also two smaller peaks present, representing minor components of $<$ 2000 and $\geq$ 500,000.

Treatment with chondroitinase ABC.

Figure 13 shows the elution profile of the same sample as that in Figure 12, after digestion with chondroitinase ABC. The material detected was reduced in concentration and the only high molecular weight material remaining eluted as a sharp peak of 22,000.

(4:4). Discussion.

The findings of this study confirm those of previous reports (King and Miller 1984), in that:

a. RMCP II was present in large amounts in the intestinal lumen of N. brasiliensis primed rats undergoing systemic shock.

b. RMCP II was secreted systemically in primed rats in a dose- (Figures 1 and 7), and time- (Figure 6) dependent manner (Figure 5) following intravenous challenge with whole worm antigen.

c. Systemic release of RMCP II was associated with the concomitant reduction of mucosal RMCP II concentration (Figures 3 and 8).

d. These changes were paralleled by depletion of mucosal mast cells from jejunal mucosa, as indicated by either **(i)** Toluidine blue stain, which detects mucopolysaccharides contained in tissues, or **(ii)** naphthol AS-D chloroacetate stain, which demonstrates the presence of specific esterases in tissue sections. In the presence of diazonium salt, enzymatic hydrolysis of ester linkages liberates free naphthol compounds which react to form highly coloured deposits at sites of enzyme activity, (Figure 3).

In addition to this, the release of GAG into plasma during this same reaction (Figures 1 and 7) was highly correlated with the release into serum of RMCP II (Figure 2), although the changes in mucosal levels of GAG did not correlate with mucosal RMCP II concentrations. The analysis of mucosal samples was however complicated by high background levels of tissue GAG and other factors likely to interfere with the assay. Nevertheless, since gut tissue was the only source of RMCP II, with the exception of lung tissue of N. brasiliensis-primed rats, which at most contains 300μ g RMCP II/g wet weight (Miller et al 1986), and since GAG release into plasma is so closely correlated with RMCP II release into serum, a common origin of these two products seems likely, with the mucosal mast cell being the most obvious source.

The systemic release of RMCP II in N.brasiliensis-primed-rats occurs immediately after challenge, with 207μ g RMCP II/ml present in serum after only 5 minutes. This increased to 439μ g/ml after

30minutes, and further increases seemed likely, if the experimental period had been extended. Previous studies (King and Miller 1984) have shown serum RMCP II levels to increase to 755μ g/ml 60min after worm antigen challenge, and the first challenge experiment of this study (in which animals were killed 60min after intravenous injection with whole worm antigen) confirmed these findings; mean serum RMCP II levels of up to 770μ g/ml were detected (Table 1).

Mean GAG levels in plasma of challenged-immune rats increased to 282μ g/ml after 30min (Table 2), and had not apparently reached a plateau (Figure 6). However, the plasma GAG levels detected in rats killed 60min after antigen challenge were in fact greatly reduced (Table 1); this was probably due to rapid clearance of GAG from the plasma in these animals. Moreover, the GAG levels detected in control animals after 60min (Table 1, groups 4 and 5) was increased ten fold, compared to animals killed after only 30min (Table 2, group 4); the longer time interval obviously allowing for greater spontaneous release of GAG into the plasma of these animals under the experimental conditions used. Thus to optimise the magnitude of the responses due to systemic anaphylaxis, a 30min time interval after antigen challenge was chosen.

The exponential increase in the mucosal mast cell number of immune rats is paralleled by increased histamine content of the jejunal mucosa. Enerback (1985) reported histamine levels in immune rat jejunal tissue (mean 20.4μ g histamine/g wet weight tissue) increased 5 fold, compared with uninfected control rats (mean 4.5μ g histamine/g wet weight tissue).

Histamine was released into plasma with similar dose-dependence as the release of GAG and RMCP II into the plasma of immune rats following intravenous challenge with whole worm antigen challenge

(Figure 7a), but no such pattern of release was detected in uninfected control animals (Figure 7b). Furthermore, there was concomitant depletion of RMCP II from gut tissue (Figure 8), and these data collectively provide strong circumstantial evidence that the measured histamine, as well as GAG and RMCP II, is derived from the mucosal mast cell, all being released during systemic anaphylaxis.

One of the most obvious differences between mucosal mast cells and connective tissue mast cells is the distinctive dye-binding properties of their granules as follows:

a. Staining with alcian blue and $MgCl_2$ (Scott and Dorling 1965) shows that mucosal mast cell granules contain lower electrolyte concentrations, and therefore less sulphated GAG than those of connective tissue mast cell granules (Miller and Walshaw 1972).

b. Spectroscopic studies of *in situ* complexes formed by GAG reacting with Toluidine blue, show spectra not typical of heparin, but of a less sulphated polysaccharide (Tas and Berndsen 1977) contained in mucosal mast cells.

c. Unlike rat connective tissue mast cells, mucosal mast cells do not exhibit the strongly fluorescent complexes characteristic of Berberine binding to heparin (Wingren and Enerback 1983).

These findings collectively provide strong evidence that the granules of mucosal mast cells do not contain heparin, but a GAG of a lower sulphate content.

Rat bone marrow derived mast cells (homologous with rat mucosal mast cells: Haig *et al* 1982; Haig *et al* 1983; McMenamin *et al* 1987) were found to contain GAG with single polysaccharide chains, the majority of which had molecular weights of the order 40,000 (Figure 9), while those of heparin are usually 7,000 - 25,000 (Robinson *et*

al 1978). The material of $\geq$ 500,000 molecular weight detected (Figure 9a) was probably proteoglycan in which individual GAG side chains had not yet been cleaved from the protein core.

A clearer indication that the GAG released from these cells is not heparin is provided by the observation that it was less sulphated than heparin, based on its Polybrene-binding affinity. All the GAG in the sample was eluted at a lower NaCl concentration than heparin, and was therefore of lower sulphate content (Figure 10). Similarly cultured rat basophilic leukaemia cells (homologous with rat mucosal mast cells: Seldin et al 1985), were found to contain non-heparin proteoglycan (Figure 11), although much of the material existed largely in macromolecular form. This confirmed previous reports of the nature of GAG contained in these cells (Razin et al 1982; Seldin et al 1985).

The GAG released into plasma during systemic anaphylaxis in the rat and the material contained in the cultured bone marrow derived mast cells, were both of 40,000 molecular weight (Figure 12). This is consistent with the mucosal mast cell origin of the increased GAG levels in plasma of infected rats challenged with whole worm antigen. Much of this material was degraded by digestion with chondroitinase ABC, and therefore had a chondroitin sulphate-like (rather than heparin-like) backbone, although some GAG resisted this treatment (Figure 13). Subsequent experiments using GAG extracted from cultured bone marrow derived mast cells have confirmed this observation, whereby chondroitinase ABC digests much of the GAG, but some undegraded material remains after this treatment. This material was likely to be heparan sulphate, but further analysis and enzyme degradation studies will be necessary to confirm this. However this does provide further evidence for the

mucosal origin of GAG released into plasma due to systemic anaphylaxis.

The findings of the studies of this Chapter have served to confirm and extend previous work on the properties and nature of GAG contained in mucosal mast cells, and highlight their involvement in the reactions occurring during systemic anaphylaxis in the rat.

Chapter 5.

Basophil Counts in Atopic and Non-Atopic Individuals

(5:1). Introduction.

The involvement of basophils in allergic disease has been recognized for many years, based on

a. their presence in tissue infiltrates occurring in various inflammatory and allergic states (Mitchell and Askenase 1983),

b. the observation that the basophil is the only blood cell to contain histamine (Sampson and Archer 1967),

c. this histamine is released in response to antigenic challenge (Lichtenstein and Osler 1964).

However, studies of their actual role and significance in allergic disease have mostly been concerned with tissue infiltrates, nasal fluids and sputum, due to their low numbers and difficulties in quantitation in whole blood (Mitchell and Askenase 1983; Denburg et al 1985; Hastie et al 1979; Okuda et al 1978; Kimura et al 1975).

Paul Ehrlich first described the basophil in 1879, and noted basophilocytosis in patients with the blood disorder chronic myelogenous leukaemia (CML). Many other workers have since confirmed his observation, and it is generally accepted that the greatest elevations of basophils occur in this disease (Forkner 1938; Doan and Reinhart 1941; James et al 1955; Juhlin 1963a; Juhlin 1963b). Basophil levels, dramatically increased in myeloproliferative disorders, particularly CML, may number more than $90,000 \times 10^6/l$ (Youman et al 1973), and can account for over 90% of the circulating leucocytes (Austen et al 1974).

Increased numbers of circulating basophil progenitors and basophils

have recently been reported in the blood of atopic patients (Denburg et al 1985): however the authors used conventional microscopic methods for cell counting, which are relatively insensitive and the emphasis of this study was in fact on basophilic progenitors. Therefore the numbers of circulating mature basophils in atopic and non-atopic individuals were determined to confirm and strengthen these findings, by using a flow cytometer - the Technicon H-6000 automated leucocyte differential analyser (Technicon, New York, USA), a modern automated method of cell counting which is extremely accurate. This method is based on preferential cellular staining (Saunders et al 1971; Ansley and Ornstein 1971), according to which leucocytes are classified by the characteristic properties exhibited by cell-specific constituents, when treated with cytochemical stains. After lysis of red blood cells, a photodetector classifies each differentially stained white blood cell by its absorbance, and/or its forward scatter characteristics.

In addition, sequential blood samples were taken from a number of both atopic and non-atopic individuals, to detect possible variations of individual basphil counts over a period of time.

(5:2) Patients, Materials and Methods.

Patients.

Control Individuals.

35 healthy adult volunteers (24 female; 11 male) of a similar age range (21 - 53; mean = 29.7) to the atopic population, were non-atopic controls. All had negative skin tests and total IgE levels of < 200 kU/l.

Atopic Individuals.

A total of 55 atopic individuals were studied; 50 were either in-patients or out-patients at the University Department of Dermatology, and 5 were atopic volunteers. Of the 50 patients studied, all had severe atopic dermatitis; 16 also had asthma, rhinitis and food allergy, 16 had asthma and rhinitis, 2 had asthma and IgE-mediated food allergy, 4 had rhinitis and food allergy, and 7 had eczema alone. Of the 5 atopic volunteers, two had asthma and rhinitis and three exhibited no allergic symptoms. The atopic patients and volunteers were of both sexes (28 female; 27 male), aged 17 to 54 (mean age = 29.6) and all produced a positive skin test (5mm wheal) and RAST score for at least one common allergen; this is generally accepted as the best working definition of atopy (Pepys 1979; Platts-Mills 1982). Total serum IgE levels ranged from 125 to 70,000 kU/l (Geometric mean = 4229.3 kU/l).

Materials.

Reagents.

RAST and IgE RIA kits for specific and total IgE determination were purchased from Pharmacia Ltd, Uppsala, Sweden. Baso acid (Product No T21-0629), Tween 20 (Product No T21-0309), baso dye (Product No T01-0628), and baso diluent (Product No T01-0555) were all purchased from Technicon, New York, USA.

Baso Acid.

This consisted of 2.18% (w/v) maleic acid with 3.75% (w/v) Tween 20.

Baso Dye.

This consisted of 0.9% NaCl,

0.143% alcian blue,

0.07% cetylpyridinium chloride (CPC),

0.7% lanthanum chloride, all w/v.

Baso Diluent.

This consisted of 0.9% (w/v) NaCl, with 0.1% (w/v) disodium EDTA.

These reagents were stable for up to one week at 4°C.

Methods.

Determination of Total and Specific IgE.

Serum samples from patient and control populations were obtained for the measurement of total and specific IgE levels. These measurements were performed by Mr HAF MacFarlane, using the following methods.

Total IgE.

Total IgE levels in serum samples were determined by double antibody radioimmunoassay (IgE RIA kit; Pharmacia Ltd). This test involved the incubation of 50µ l of test serum or dilutions of the test serum in IgE-free diluent (0 units/ml standard; Pharmacia), with 50µ l of ^{125}I- labelled human IgE and 50µ l of rabbit anti-human IgE in polystyrene tubes. This was incubated at 22°C for 3h, when a second antibody was added (2ml of sheep anti-rabbit IgG coupled to Sepharose gel), and then incubated for a further 30 minutes at 22°C.

Bound radioactivity, which was indirectly proportional to the concentration of IgE in the serum samples was measured using a NE

1600 γ -counter. Standard serum containing 0, 2, 5, 15, 100, 400 and 1000 units of IgE/ml was used for the standard curve. All test and standard samples were assayed in duplicate, and mean counts were used in all calculations. The values of the samples were read from a graph of concentration of standard (log) vs % counts bound (linear).

Allergen-Specific IgE.

Measurement of the levels of allergen specific IgE in serum samples was by the Radio Allergosorbant Test (RAST; Pharmacia Ltd). Sera were incubated with a paper disc to which a particular allergen had been covalently coupled. 50μ l of the test serum or dilutions of the test serum in RAST buffer (Pharmacia) were incubated with an allergen disc for 3h at 22°C; any allergen-specific IgE in the serum sample would at this stage bind to the paper disc. After incubation, 2ml of 0.9% NaCl containing 1.0% Tween 20 (w/v) was added to each tube and left for 10 minutes. This washing solution was then removed from the paper discs by suction, and the washing process repeated twice. The paper discs were finally dried using a Pasteur pipette attached to a vacuum line. 50μ l of ^{125}I-labelled anti-human IgE was added, and incubated for 24h at 22°C. The paper discs were washed as before, and counted in a γ -counter. The amount of radioactivity was directly proportional to the amount of allergen-specific IgE on the paper disc.

Four reference sera and reference discs were used to quantify the results.

Reference Serum.	Phadebas RAST units/ml (pru/ml).	Grading.
A.	> 17.5	4 Very strong positive.
B.	3.5 - 17.5	3 Strong Positive.
C.	0.7 - 3.5	2 Moderate.
D.	0.35 - 0.7	1 Weak Positive.
	> 0.35	0 Negative.

The results for each of the standard sera were used to plot a standard curve, from which the test results were read. Test samples with values of > 12 pru/ml were diluted and reassayed.

Determination of Cell Counts.

These measurements were performed by Dr TL Allen and Mr F MacKenzie, using the following methods.

Whole blood samples (5ml) were taken into EDTA (0.025M) vacutainer tubes, and diluted with an air segmented stream of baso diluent, to which baso dye was then added. The use of CPC caused red blood cell lysis, eliminated undesired precipitates, and prevented the clumping of red cell membranes. Lanthanum chloride selectively inhibited the staining of nucleic acids. Baso acid was added in two steps to avoid precipitation, and only basophils were stained. The analytical stream then entered a sheath-stream flow cell where the forward-scattering signal of each cell was measured at two non-overlapping wavelengths. The optical measurements were then

converted into electrical pulses by photodiodes and applied to the logic circuits for cell classification and quantification. As each blood sample passed through the machine, 20,000 white cells were individually characterised by means of their light absorbance and scattering properties, and according to their cytochemical staining.

(5:3). Results.

Circulating Basophil Counts.

Absolute numbers of circulating blood basophils of both atopic and non-atopic populations are shown in Figure 1, with the median values given for both populations. Using the Mann-Whitney U test, the atopic population had significantly greater numbers of circulating basophils (median= $70x10^6/l$), than the healthy controls (median= $50x10^6/l$), ($p=0.0005$). There was a correlation between the number of total leucocytes and the number of basophils in the non-atopic individuals ($r=0.51$; $p= 0.0014$), but not in the atopic population ($r=0.081$; p=NS). However there was no correlation of basophil counts with age, total IgE levels, or clinical symptoms in either population.

Eosinophil Counts.

Absolute numbers of eosinophils of both atopic and non-atopic populations are shown in Figure 2, with the median values given for both populations. Using the Mann-Whitney U test, the atopic population had significantly greater numbers of circulating eosinophils (median= $520x10^6/l$), compared with the non-atopic controls (median= $140x10^6/l$), ($p< 0.001$). The number of eosinophils and basophils correlated in both the atopic ($p< 0.02$), and non-atopic ($p< 0.0002$) groups.

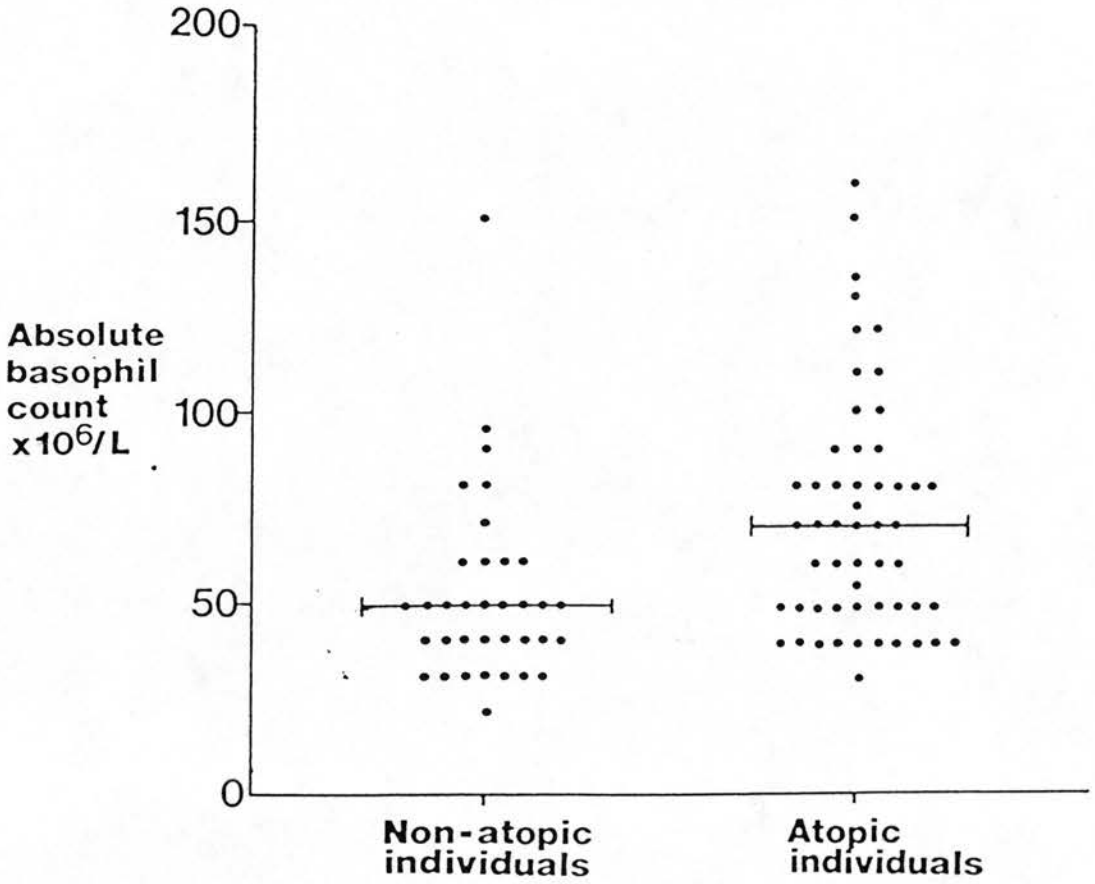


Figure 1. Numbers of circulating basophils were overall significantly greater in the atopic population (median = 70 x 10^6/l), than in the non-atopic group studied (median = 50 x 10^6/l), (p = 0.0005).

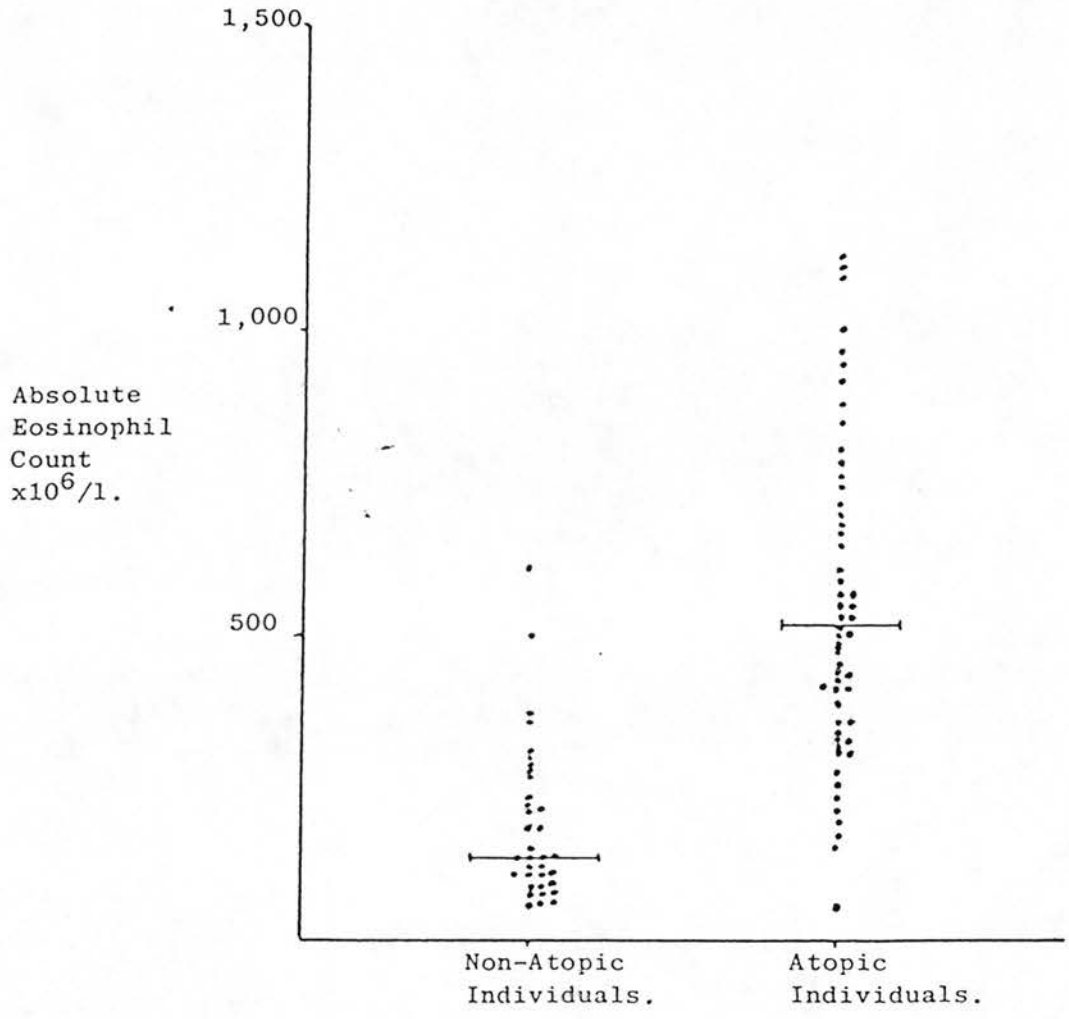


Figure 2. Numbers of circulating eosinophils were overall significantly greater in the atopic population (median = 520 x 10^6/l), than in the non-atopic group studied (median = 140 x 10^6/l), ($p < 0.001$).

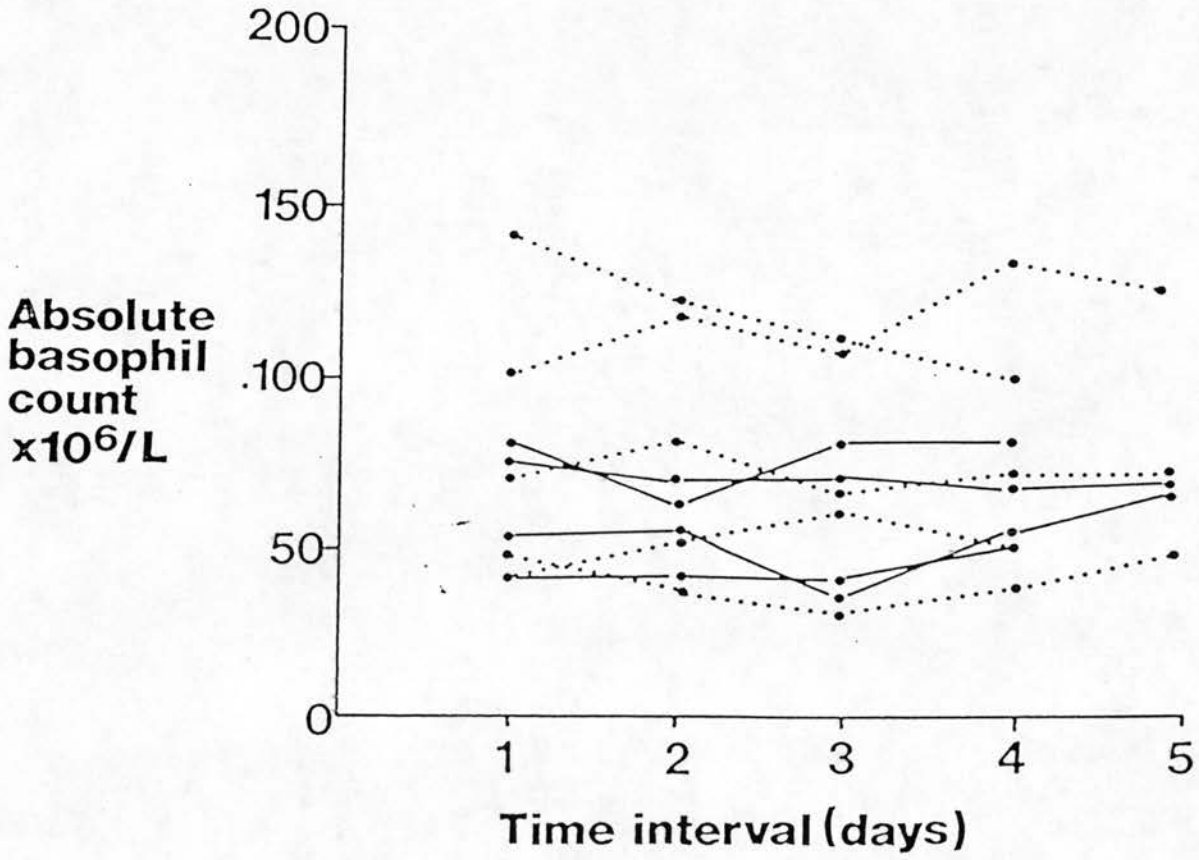


Figure 3. Basophil counts were obtained from five atopic (●---●) and four non-atopic (●——●) individuals at the same time over a period of four or five days.

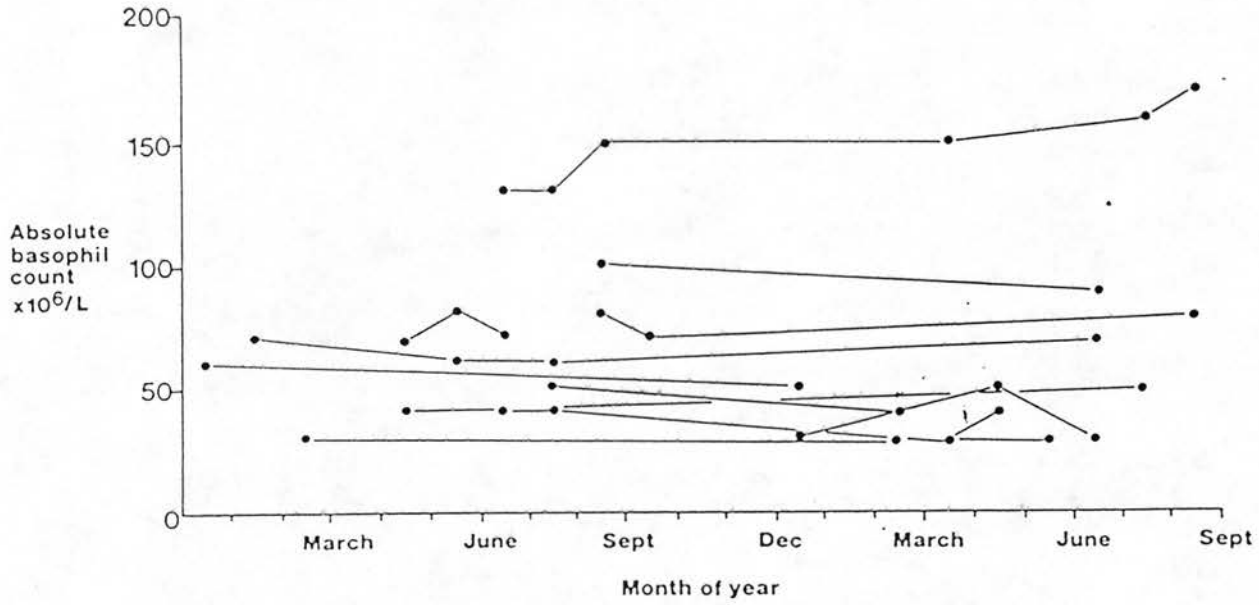


Figure 4. Basophil counts were obtained from both atopic and non-atopic individuals at various time intervals, over a period of up to twenty months.

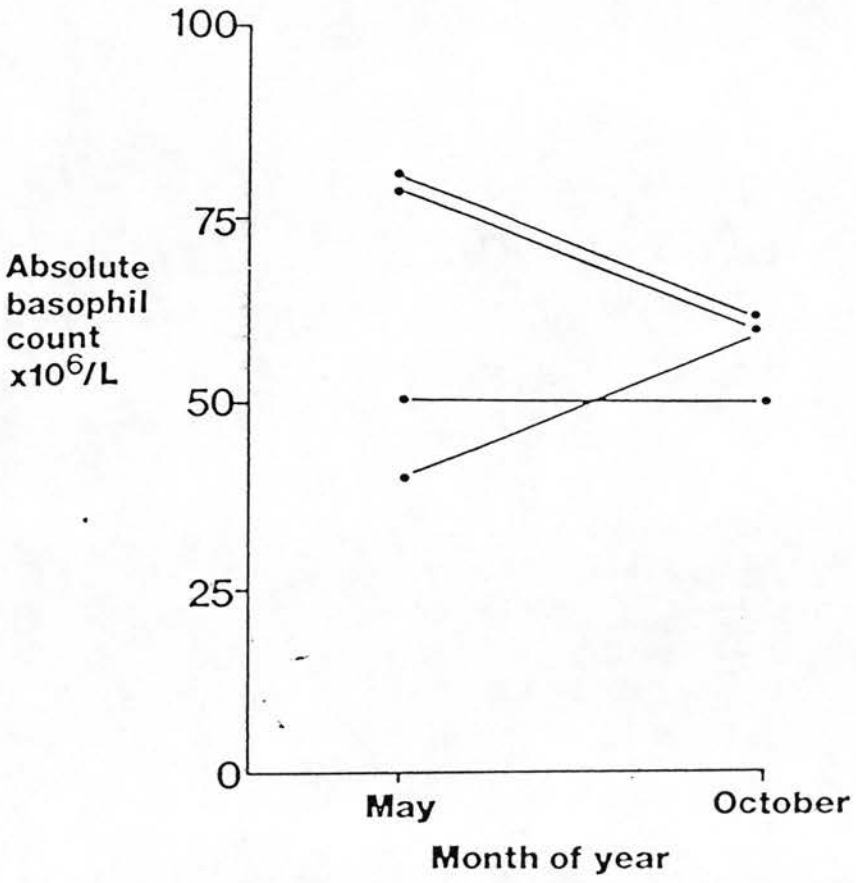


Figure 5. Basophil counts were obtained from four atopic dermatitis patients who also had seasonal rhinitis due to grass pollen. Samples were taken over a time interval of six months.

Daily Variation of Basophil Counts.

Basophil counts were obtained from five atopic and four non-atopic individuals at the same time on either four or five consecutive days. There was no significant day to day variation in either group (Figure 3).

Monthly Variation of Basophil Counts.

Basophil counts were obtained from 6 atopic and 5 non-atopic individuals at various intervals over periods of up to 20 months (Figure 4). Again there was no significant variation in the basophil counts within individuals in either group.

Seasonal Variation of Basophil Counts.

Four patients from the atopic group, known to have seasonal rhinitis due to grass pollen, were studied at various intervals over a period of six months (Figure 5), and no obvious pattern of seasonal variation was detected.

Circulating levels of basophils showed little variation within individuals on both a short term and long term basis.

(5:4). Discussion.

The observation that mature circulating basophil levels are elevated in atopic individuals, using this new accurate method for cell counting, confirms the findings of Denburg et al (1985), who used a less accurate and less sensitive conventional microscopic method. This typically involves the examination of 500 leucocytes on a Romanovsky stained blood film, and since basophils occur relatively infrequently, gives rise to a large inherent statistical error. Other errors may arise from inadequate sample mixing, poorly spread peripheral blood film, or inaccurate identification of basophils.

The Technicon H-6000 differential leucocyte count is based on a count of approximately 20,000 cells (depending on total white cell count), and thus has much greater precision, and a very marked reduction in the co-efficient of variation of replicate analyses (CV). The variance inherent in these two basophil counting techniques was examined by Winkel (1976), who showed that visual basophil counts had a CV of 141%, whereas Technicon H-6000 basophil counts had a CV of 13.1%.

Some consideration has to be given to the accuracy of basophil counts in general terms. The most obvious major problem is that there is no quality control material with an accurately assigned absolute basophil count available. It follows therefore that whatever method of basophil counting is chosen, it is not possible to calibrate the method using a reference preparation. Bearing this in mind, the method chosen for basophil counting must be based on sound scientific principles, and must at the very least be precise. In this respect therefore, the Technicon H-6000 analyser is by far the most superior method for the enumeration of basophils in whole blood.

The increased absolute number of basophils in the atopic group correlated with the percentage of basophils in each differential leucocyte count ($r = 0.83$; $p < 0.005$), but did not correlate with the total leucocyte count ($r = 0.081$; $p = NS$). Thus the increased number of basophils is not merely due to an increase of total leucocytes in this group, as in the non-atopic population, where basophil counts paralleled the total leucocyte counts ($r = 0.61$; $p < 0.002$).

Increased levels of circulating eosinophils were detected in individuals in the atopic group (Figure 2). The observation that

basophil and eosinophil counts correlate in both groups studied (control group $p< 0.005$; atopic group $p< 0.002$), is in keeping with previous reports where their levels in peripheral blood have been found to fluctuate in parallel, in response to a variety of antigenic and hormonal stimuli (Dvorak et al 1974a; Ogilvie et al 1980; Rothwell 1975; Rothwell and Love 1975). It is well recognized that eosinophil counts are also raised in atopy (Weller 1984), and their role is thought to complement that of basophils, involving the production of various chemotactic factors during inflammatory reactions (Weller and Goetzl 1979). Furthermore, they may function synergistically in certain states, for example, in immune reactions to parasites (Brown et al 1982). It is not surprising therefore to find that basophilia occurring in certain clinical conditions is accompanied by raised levels of circulating eosinophils.

Although the half life of the basophil is 2-5 days (Mitchell and Askenase 1983), it is believed to spend only about 12 hours in circulation (Gleich et al 1986). The observation that actual numbers in the circulation of specific individuals varies relatively little (Figures 3-5), indicates that this routine production and destruction of basophils is closely regulated.

Hirsch and Kalbfleisch (1976) reported fluctuating levels of circulating basophils in a number of individuals with hay fever, again using traditional visual methods for cell counting. These individuals, who had been carefully selected as clinically sensitive only to ragweed allergen, showed a significant elevation of basophils during the symptomatic period of the ragweed season. The apparent lack of seasonal variation of basophil counts observed in the present study (Figure 5) may have been due to the multiple allergies of the patients, obscuring any seasonal effect on the

production of basophils. However, the variation of basophil counts in the individuals of this study was very slight, and seemed to be independent of allergic state, day of the week, or month of the year, and the conflict of results with those of Hirsch and Kalbfleisch (1976) may equally result from the use of the Technicon H-6000 and increased accuracy of cell counting.

Factors controlling production of basophils in the bone marrow have not been well established, although their similarity to mast cells, which also have a myeloid origin, may provide some clues. Factors regulating mast cell production and maturation are thought to be T-cell derived (Morley et al 1984). For example, interleukin 3 causes maturation of tissue mast cell precursors, and lymphokines are well established as regulators of the allergic response; the source of both are T-helper lymphocytes. Lymphocytes are also known to regulate delayed hypersensitivity reactions, in which basophil accumulation may occur (Denburg et al 1980), and thus similar factors may regulate the production of basophils.

Although certain functions have been identified for the basophil in vitro (Lichtenstein et al 1986), and in vivo, such as host defence and resistance to parasites (none of which were relevant to the individuals involved in this study), the overall functional integrity of basophils has yet to be evaluated. Equally, the role of the basophil in IgE-mediated hypersensitivity, and therefore atopy, still remains to be defined, but the observation that there are increased circulating basophil levels in atopic individuals, underlines that this role is likely to be of considerable importance.

Chapter 6.

Allergen-Induced Release of GAG and Histamine from Human Basophils.

(6:1). Introduction.

Anaphylactic type hypersensitivity reactions are initiated when an appropriate antigen binds to and cross-links IgE molecules on the surface of mast cells or basophils. This results in the non-cytotoxic release of preformed mediators stored in the cellular secretory granules (Schwartz and Austen 1984). When released, these preformed secretory components, which include histamine, heparin-like proteoglycan, chemotactic peptides and a range of enzymes, elicit the acute reactions characteristic of immediate hypersensitivity. Mast cells are the major source of tissue histamine (Riley and West 1953), and basophils of humoral histamine (Graham et al 1955), the amine being stored in the granules in association with the acidic groups of heparin-related GAGs and/or proteins by means of ionic bonds (Uvnas et al 1970).

The development in recent years, of several different methods for the measurement of total and allergen-specific serum IgE, was an important improvement in the diagnosis of allergic states. However, these parameters provide little information as to the precise mechanisms of allergic reactions: immediate hypersensitivity reactions in man or in experimental animals do not depend directly upon circulating IgE antibodies, but upon the release of the mediators from IgE-sensitized mast cells and basophils. Thus, in vitro methods of diagnosis should reflect the end stage of the reaction, ie. the degranulation of the cells

involved. The diagnosis of allergies based on cellular reactivity has been the subject of research for many years (Shelley and Juhlin 1961; Klopstock et al 1962; Hirsch and Zastrow 1972) and it is therefore not surprising that the measurement of histamine release from basophils is well established and has been extensively used for in vitro studies of human allergy (Siraganian and Hook 1976b). However, GAG release from basophils has not been previously studied in this way. The studies outlined in this Chapter have used the competitive binding assay for GAG and standard histamine assay (both of which are described in Chapter 2) to study further and compare the properties of antigen mediated release of these compounds from human basophils in vitro.

(6:2) Patients, Materials and Methods.

Patients.

Atopic Individuals.

Fourteen atopic subjects were studied in total, 10 patients with atopic dermatitis and 4 atopic volunteers. Patients studied were either in-patients or out-patients at the University Department of Dermatology, Royal Infirmary of Edinburgh, and were of both sexes (5 female; 9 male) and aged 19-46 years (mean age = 31 years). Of the atopic dermatitis patients, 9 also had asthma, 7 had rhinitis and 5 had known food allergy. One of the atopic volunteers had asthma and perennial rhinitis, one had had asthma as a child, and 2 exhibited no allergic symptoms. All produced a RAST score of 4 for house dust mite allergen (D.pteronyssinus specific IgE range, 34-1750 RAST units: 1 unit approximately equivalent to 1KU total IgE/l), and had total IgE in the range of 483 - 67,200 KU/l.

Control Individuals.

Eight healthy adult volunteers (6 female; 2 male) of a similar age range (23-34 years; mean age 28 years) to the atopic population, were non-atopic controls. All had negative skin test results, total IgE levels of < 200 KU/l, negative skin test results, and a RAST score of 0 for house dust mite allergen.

Materials.

Reagents.

Freeze dried preparations of D.pteronyssinus were purchased from Pharmacia, Uppsala, Sweden, and reconstituted in 0.9% (w/v) saline solution to obtain stock solutions of 10μ g/ml of allergen, which was further diluted using Tris ACM buffer for release experiments.

Buffers.

Tris ACM buffer.

This consisted of Tris A buffer (described in Chapter 2), supplemented with 0.6mM $CaCl_2$ and 1mM $MgCl_2$.

Methods.

Preparation of leucocytes.

Leucocytes were prepared essentially as described by Siraganian and Hook (1976b), and is described in detail in Chapter 3. The leucocyte-rich cell pellet was finally resuspended in Tris ACM buffer. This method produced cell fractions of 3.4 - 5.9% purity with little loss of total basophil numbers.

Release experiments.

These were performed using 1.5 ml of cell suspension, with 150μ l of allergen diluted in Tris ACM buffer. After 60min at 37°C, samples

were centrifuged (110 x g; 15min; 4°C) and supernatants assayed in duplicate for both GAG and histamine.

Ca^{++} Requirement.

To determine the Ca^{++} requirement for antigen induced release, 40 ml of whole blood from four atopic individuals were sampled into dextran/ dextrose/ EDTA as before, and at the same time a further 40 ml sampled into dextran/ dextrose/ EGTA (12.5mM). All samples were left to sediment for 90 minutes, and leucocytes prepared as before. Release experiments were performed in the absence and presence of 0.6 mM Ca^{++} and 1mM Mg^{++}, using D.pteronyssinus at a concentration previously found to cause release of both GAG and histamine.

Determination of Total Histamine Content of Cell Preparations.

To determine the total histamine content of the cell preparations used, basophil enriched fractions were prepared as before from atopic individuals, and treated with 12% perchloric acid to obtain complete cell lysis. After centrifugation, supernatants were assayed for histamine.

Determination of Basophil Counts.

Whole blood and cellular suspensions were stained, and the number of basophils in each sample was determined by the methods described in Chapter 5.

Assay of Histamine.

The extraction and assay of histamine was performed by the manual fluorometric method described in Chapter 2:2.

Assay of GAGs.

Highly sulphated GAGs were measured in each sample by the method described in Chapter 2:1. Interference in the assay from membrane components of concentrated cell lysates precluded determination of total cell GAG for each individual studied. Results were therefore expressed as a percentage of spontaneous or background release, when no allergen was present (background = 100%). Since a comparison with histamine was to be made, its release was expressed in the same way.

Statistical Analysis.

Statistical analysis was performed using the paired Student's t-test.

(6:3) Results.

Ca^{++} Requirement.

The Ca^{++} requirement for allergen-induced release of GAG and histamine from cells of 4 atopic individuals is shown in Table 1. In the presence of EDTA, which sequestrates both Ca^{++} and Mg^{++}, release of both GAG and histamine was very low; with EGTA, which removed existing stores of Ca^{++} but not Mg^{++}, release was only slightly increased. Maximum release occurred in the presence of both Ca^{++} and Mg^{++} added at concentrations of 0.6mM and 1mM respectively.

Total Histamine Content.

The mean total histamine content of cellular preparations was 60.3 (range = 42.0-75.2; SEM=32.0) ng/ml, which was comparable to the

Conditions.	25mM EDTA	25mM EGTA	0.6mM Ca^{++} 1.0mM Mg++
Histamine Released			
ng/ml (mean ± SEM)	13.7 ± 1.42	19.7 ± 0.93	37.0 ± 2.19
GAG Released			
ng/ml (mean ± SEM)	267.7 ± 43.2	294.0 ± 60.3	803.7 ± 80.0

Table 1: **Dependence of Release on Divalent Cations.**

Cell fractions were obtained from four atopic individuals in the presence of EDTA (0.025M, to remove existing stores of Ca^{++} and Mg^{++}), or EGTA (12.5mM, to deplete stores of Ca^{++} only). To show the Ca^{++} requirement for release, cell fractions were supplemented with Ca^{++} (0.6mM) and Mg^{++} (1mM). Values are expressed as mean and SEM.

maximum histamine values obtained with D.pteronyssinus-induced release (mean=42.5; range=10.5-95.0; SEM=11.37ng/ml).

Dose Response of Allergen-induced Release of GAG and Histamine from Basophils.

D.pteronyssinus was added to cell suspensions at five different dilutions and dose response relationships were observed between the concentration of allergen and the release of histamine and GAG in atopic subjects. However the dose response peaks of histamine and GAG release were apparently dissociated in all cases; this is illustrated in Figure 1. This individual was studied twice, with a six month interval between tests, and the release patterns obtained were similar on each occasion. There was however wide individual variation in allergen induced release patterns. Levels of release at each concentration of D.pteronyssinus were therefore expressed as % of spontaneous or background release for each individual (background = 100%) to indicate magnitude of response. Mean values of allergen-induced histamine and GAG release for both groups studied are shown in Figure 2. Mean background histamine release was 11.08 (SEM=1.82) ng/ml for the atopic individuals, and allergen-stimulated release increased with dose to 372.4 (SEM 99.5)% of this level at a D.pteronyssinus concentration of 10μ g/ml. By contrast, maximum release of GAG occurred on average at 0.1 μ g/ml D.pteronyssinus, and amounted to 227 (SEM=63)% of the mean background GAG release of 463 (SEM=63) ng/ml. GAG release above background was significant at the $p < 0.001$ to $p < 0.05$ level for the atopic group.

Although there was no allergen-induced release of histamine from cells of non-atopic individuals (background release = 9.2ng/ml, SEM

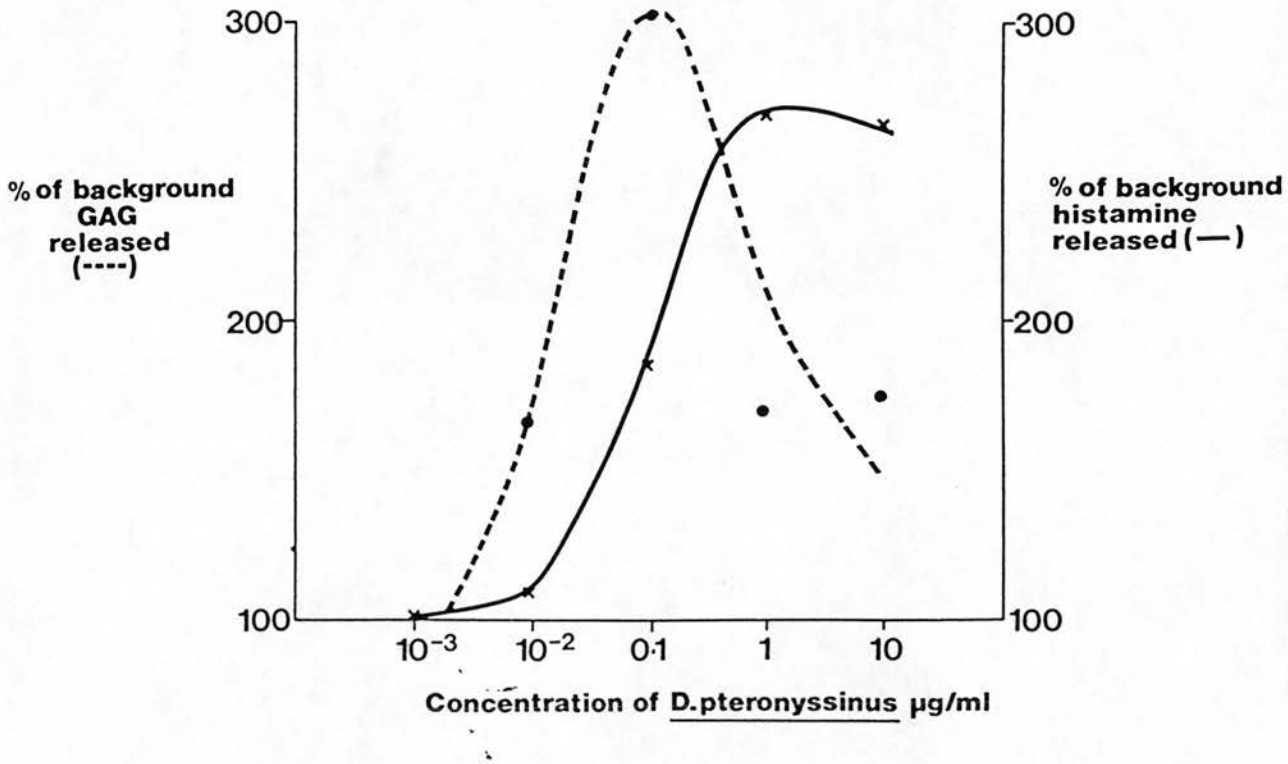


Figure 1: Release of GAG and Histamine in an Atopic Individual.

Release of GAG and histamine were measured from cells challenged with five concentrations of allergen. Each point represents the mean of 2 determinations. This illustrates the pattern of release from one of the atopic patients studied. Results obtained at each allergen concentration were calculated in relation to spontaneous or background release (spontaneous release of GAG = 345ng/ml, and histamine=20ng/ml), using these values as 100%.

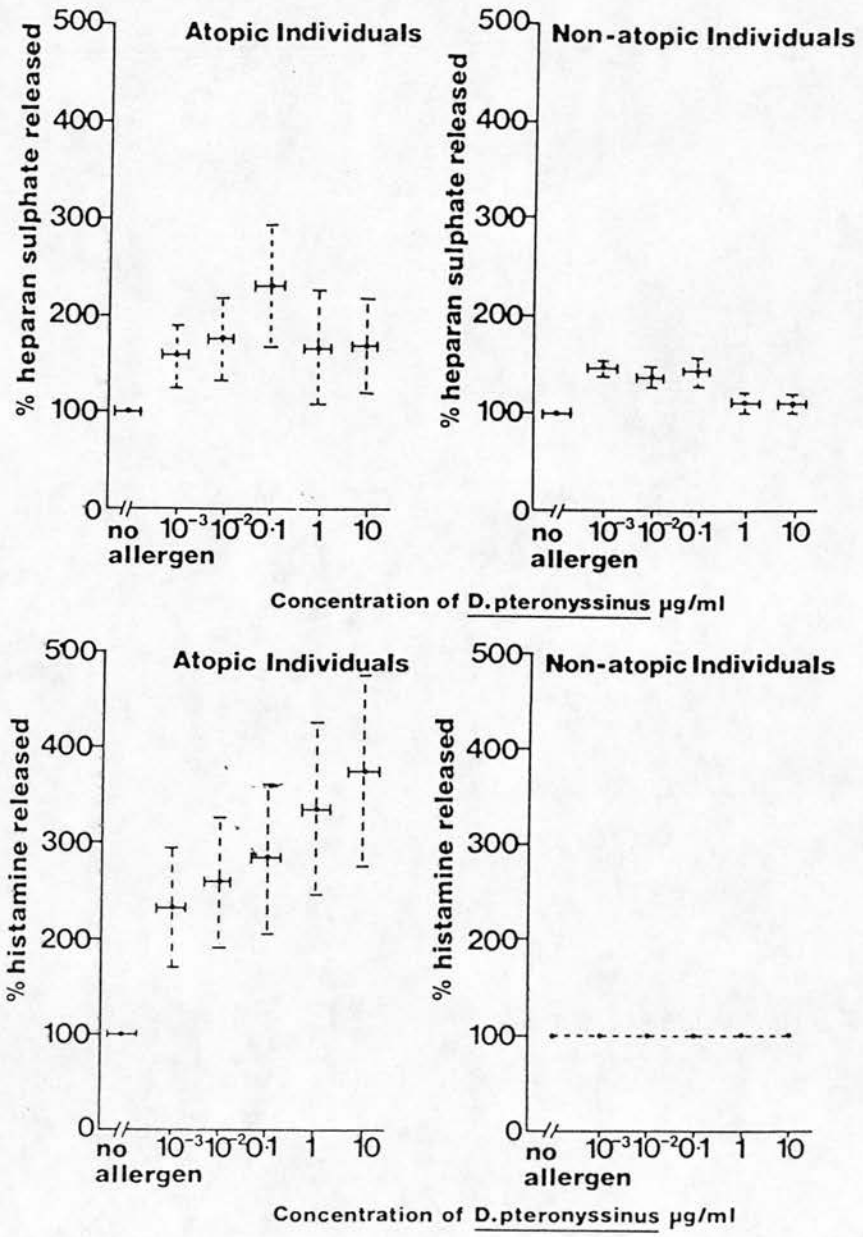


Figure 2: Release of GAG and Histamine in Atopic and NON-Atopic Individuals.

Release levels are shown as mean values with SEM for each allergen concentration within each population. Release of GAG was significantly raised above background for both populations at each allergen concentration ($p < 0.01$ to $p < 0.05$, and $p < 0.001$ to $p < 0.05$ for atopic and non-atopic groups respectively). Mean basophil concentration for non-atopic individuals was 59 (SD=38)$\times 10^6$/l, and 67 (SD=24)$\times 10^6$/l for the atopic group. Release of histamine from the non-atopic group was less than or equal to background release.

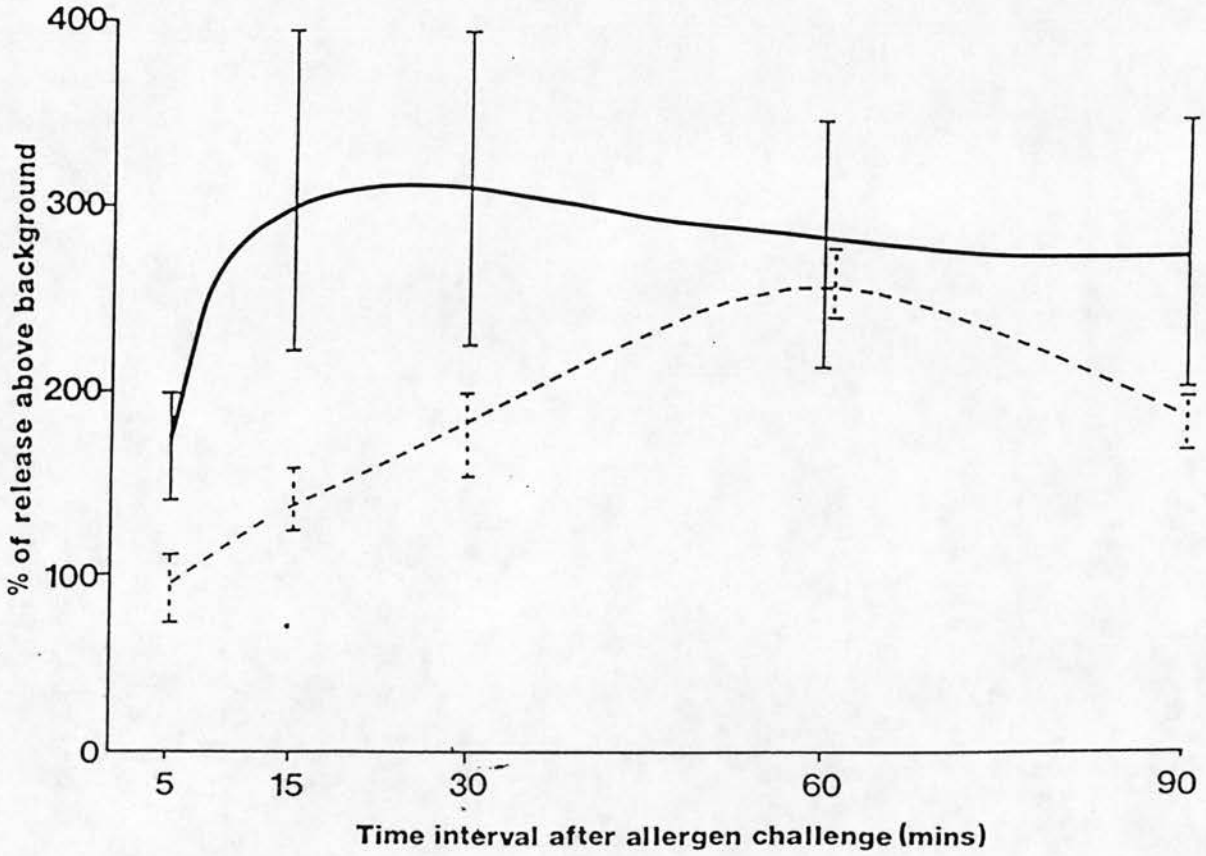


Figure 3: **Time Course of GAG and Histamine Release in Atopic Individuals.**

Aliquots of cell suspension were removed at various time intervals after addition of allergen, and levels of GAG (·----) and histamine (——) release were measured. Release was calculated as % of background for each individual, (mean background GAG release = 447.5 (SEM=61.3)ng/ml, and mean background histamine release = 18 (SEM=5.48)ng/ml) using these values as 100%, and plotted with mean and SEM for each time interval.

= 2.18ng/ml), some release of GAG above background of 526.3 (SEM = 66.9) ng/ml was observed with these cells; this was significant at the level $p< 0.01$ to $p< 0.05$, and reached a maximum of 148% above background at a D.pteronyssinus concentration of 10^{-3} μ g/ml.
The basophil concentrations were 67 (SEM=6.4) x 10^6/l for the atopic subjects, and 59 (SEM=13.4) x 10^6/l for the non-atopic controls.

Time Course of GAG and Histamine Release.

Leucocytes from 6 atopic individuals were challenged with D.pteronyssinus allergen concentrations previously found to cause release of both GAG and histamine for each individual. Aliquots of cell suspension were removed 5,10,15,30,60 and 90min after allergen challenge. Each aliquot was centrifuged as before and assayed for both GAG and histamine. GAG release was slow and required 60min incubation with allergen to complete, whereas maximum histamine release occurred within 15min of allergen challenge (Figure 3). Results are expressed as % background release to show magnitude of response obtained; mean background release of histamine was 18.0 (SEM=5.48) ng/ml, and mean background release of GAG was 447.5 (SEM=61.3) ng/ml.

(6:4). Discussion

The study of the role of the basophil in allergic disease to date has been hindered by their small numbers in whole blood, and the limited number of tests of basophil function. Of the tests available, the release of histamine from the secretory granules is most widely applied (Siraganian and Hook 1976b). Since histamine is

known to be associated with heparin-like molecules in cytoplasmic granules (Galli and Dvorak 1979), the GAG assay described in Chapter 2:1 was used to evaluate the characteristics of its release from basophils in the allergic response.

Since GAG was previously found to be exclusively contained in the basophil fraction of whole blood (Chapter 3), and this is also the case for histamine (Graham et al 1955), it may be concluded that although whole blood or leucocyte preparations were used in the experiments of this Chapter, release of these compounds indicated the response of the basophils, and no other cell type.

Release of GAG and histamine were similarly inhibited by Ca^{++} and Mg^{++} chelation with EDTA, and sequestration of Ca^{++} alone with EGTA; release of both mediators thus exhibited a common Ca^{++} requirement. Mg^{++} alone did not support release. It is generally assumed that since histamine and GAG are contained in the same granules within cells, they will be released together during degranulation. However, this is the first study in which allergen-induced GAG release from basophils has been examined in detail, and the evidence suggests that they are released at different stages of the degranulation process, possibly by different mechanisms. Optimum release of histamine from cells of atopic individuals occurred within 15min of allergenic challenge, whereas GAG release from the same cells occurred at a slower rate, requiring at least 60min to complete. Histamine is already known to have different time courses of release, depending on the stimulus initiating the degranulation process (Lichtenstein and Osler 1964; Siraganian and Hook 1977), and there have been previous reports of various other mediators being released at different rates (Theohardies et al 1982), which has been explained by the possible

existence of different sites of mediator storage (Tamir et al 1982). Alternatively, the different time courses of GAG and histamine release may be explained by the theory of Uvnas (1978), which postulates the formation of a "pore" when fusion between the secretory granule membrane and plasma membrane occurs at degranulation. This pore might then act as a dialysing membrane across which ion exchange mechanisms are thought to result in the release of small molecules such as histamine. Release of the macromolecular GAGs would be slower and occur at a later stage of the degranulation process.

Release of both GAG and histamine from basophils of atopic individuals could be stimulated by D.pteronyssinus allergen. However a wide range of allergen-induced release patterns were obtained, and optimum release of histamine and GAG occurred at different allergen concentrations for each individual. Although release of GAG occurred more slowly than that of histamine, the allergenic stimulus required to elicit maximum release of GAG was less. Again this points to the existence of a complex release mechanism, possibly involving more than one compartment.

A smaller but significant amount of GAG release was also detected in the non-atopic group. This occurred independently of histamine release and was probably due to a non-immunological response. A large number of factors are known to cause degranulation of mast cells and basophils, including various chemicals such as aspirin (Asad et al 1984), various components of complement (Siraganian and Hook 1976), lectins (Foreman et al 1977), physical stimuli such as cold (Soter et al 1976), and spontaneous release has been reported from cultured human colonic mucosa (Elakim et al 1986). The D.pteronyssinus preparation used in release experiments may contain

some agent capable of promoting a small amount of non-IgE mediated release of GAG from basophils of non-atopic individuals.

It has long been recognized that GAG is released from basophils on degranulation, but in the absence of a sufficiently sensitive assay this process has not been examined in detail. Further investigation is needed to elucidate the exact mechanisms involved, but the preliminary findings of this Chapter indicate that the properties of the Ca^{++}-dependent release of GAG from human basophils is different from that of histamine in several respects, and may occur, at least in part, by a different mechanism.

Chapter 7.

Human Mast Cells in Nasal Turbinates of Atopic and Non-Atopic Individuals.

(7:1). Introduction.

Studies of human mast cells frequently utilise nasal tissue for a number of reasons.

a. Mast cells are abundant in the nasal mucosa and are easily recognized in toluidine blue stained biopsy sections. They occur frequently in the sub-epithelial layer, occasionally between epithelial cells, and surrounding blood vessels in the deeper layers of the lamina propria (Wihl 1979).

b. Nasal tissue is easily obtained, and biopsies can be taken without anaesthesia.

c. Nasal secretions contain granule-derived mediators, and reflect the extent of degranulation of the mast cells; repeated samples of fresh tissue, secretions and smears are easily obtained, and intact mucous membranes are easy to inspect.

The frequency of mast cells in the nose is thought to aid in the defence against harmful airborne agents, thus the nose filters, warms and moisturises inspired air, as well as functioning as an olfactory sense organ. However, many airborne agents are inhalant allergens, such as grass and weed pollens and house dust mite allergen; these provoke IgE-mediated allergic reactions in sensitized individuals, and result in the symptoms of allergic rhinitis. These symptoms can also be produced by food, beverages, non-specific agents in the ambient air, and changes in temperature, the severity of which are dependent upon the reactivity of the

nasal mucosa of individuals (Borum 1979).

Release of mediators from mast cells in nasal allergy causes sneezing, rhinorrhoea, nasal obstuction, mucosal oedema, itching of the nose, palate and pharynx, and the accumulation of eosinophils in the tissues.

The importance of the role of histamine in the pathogenesis of such reactions is well recognized and is reflected in the benefits of antihistamine therapy (Mygind 1982); these drugs successfully control itching and sneezing, but often fail to relieve obstruction, in which case surgery may be required.

In this Chapter, nasal tissue from atopic and non-atopic individuals was used to study the content of histamine and GAG in human mast cells as a marker for mast cell involvement in atopic disease, and as a comparison with the other studies described, which are mostly concerned with basophils.

(7:2). Patients and Methods.

Patients.

Twenty six patients were studied in total (16 male: 10 female) all undergoing bilateral inferior turbinectomy for chronic rhinitis. Of these patients, 15 were non-atopic (aged 13-72 years; mean=41 years) and 11 were atopic (aged 16-64 years; mean=27 years). Atopic history, principal symptoms and differential leucocyte counts were noted. A diagnosis of atopy was based on standard prick skin tests to 11 common allergens (a weal of 4mm or greater, present at 15 minutes to one or more allergen signified a positive result), and measurements of total serum IgE, and IgE specific for 3 common inhaled allergens (house dust mite, grass pollen and animal dander). One patient was not skin tested because of severe eczema.

Methods.

Preparation of Tissue Samples.

On removal, 1 turbinate (right or left by random allocation) was placed in 10ml 0.9% (w/v) NaCl at 4°C, and within 3-5 minutes all bone was removed and tissue coarsely chopped into 2-3mm fragments, followed by light centrifugation for 5 minutes to separate solid tissue fragments and supernatant fluid. The supernatant fluid samples containing free GAG and histamine, and the nasal tissue containing the cellular mediators were then stored separately at -20°C for subsequent GAG and histamine estimation. Prior to biochemical analysis, 0.9% NaCl was added to the nasal tissue fragments to standardize weight/volume ratios in all samples, which were then homogenized at 20,000 rpm for 50 seconds.

Determination of Total and Specific IgE.

These measurements were made using the methods described in Chapter 5.

Assay of Histamine.

The extraction and assay of histamine was performed by the manual fluorometric method described in Chapter 2:2.

Assay of GAG.

Highly sulphated GAGs were measured in each sample by the method described in Chapter 2:1.

Assay of Protein.

The protein content of the homogenized tissue specimens was measured by Mr D Pratt, MRC/SNBTS Blood Components Assay Group,

Edinburgh, using a dye binding method (BioRad Protein Assay, England) with bovine serum albumin as the standard.

Mast Cell Counts.

The second turbinate removed from each patient during surgery was used for microscopic examination of tissues, and to obtain mast cell counts. These measurements were performed by Dr DM Salter, Department of Pathology, University of Edinburgh, who also provided the electron micrographs. Three transverse blocks were taken and fixed in Carnoy's solution and 3μ m paraffin sections were stained with 1% toluidine blue. Cell counts were performed with an eyepiece graticule ($0.23mm^2$) at a magnification of x 250. Ten sites were randomly selected from each of the 3 blocks. At these 10 sites the edge of the graticule was orientated at the epithelial surface and 3 contiguous areas perpendicular to the surface counted. Counts were expressed as number of cells per mm^2, a total area of 6.9 mm^2 having been counted. Additional counts were made on serial sections of turbinates fixed in 4% neutral buffered formaldehyde. These were stained with either 1% toluidine blue or with polyclonal anti-IgE (Dakopatts Ltd, Denmark) using a standard peroxidase (PAP) technique. In a few atopic and non-atopic subjects, electron microscopy was performed to assess the degree of degranulation of the mast cells.

Statistical Analysis.

Statistical analysis was performed using the Student's t-test on biochemical data, and the Wilcoxon rank sum test on the mast cell counts. Correlation coefficients were used to establish the relationship, if any, between the variables measured.

Histamine and protein values are expressed as mean ± standard deviation. Total IgE and GAG levels failed to show a normal distribution and these data were log transformed prior to analysis, and geometric means derived.

(7:3). Results.

Clinical Details and Symptoms.

The clinical symptoms of the patients studied are shown in Table 1. As expected, nasal obstruction was the commonest symptom (24 patients). Rhinorrhoea and sneezing were more common to the atopic group, and facial pain to the non-atopic group.

The total serum IgE levels were significantly raised in the atopic group (geometric mean 200KU/l), when compared with those of the non-atopics (geometric mean 19KU/l), ($p < 0.001$). RAST scores correlated with skin test results in all but one patient.

Histamine, GAG and Protein Content of Nasal Tissue and Supernatant Fluid.

Histamine, GAG and protein concentrations of both groups are summarised in Table 2.

Protein Content

Protein concentrations in the tissue homogenates did not differ between the two groups.

Histamine Content.

There was no significant difference between atopics and non-atopics in either free or cellular histamine levels (Figure 1a), or in the ratio of free to cellular histamine (Table 2). Histamine levels did

Table 1: Clinical Symptoms.

	Non-atopic Individuals. n = 15	Atopic Individuals. n = 11
Obstruction	15	9
Sneezing	14	9
Rhinorrhoea	14	9
Facial pain	13	5
Anosmia	10	4

Table 2: Histamine, GAG and Protein Content of Nasal Tissue and Supernatant Fluids.

		Non-atopic Individuals.	Atopic Individuals.	
*Histamine	Free (ng/ml)	32.6 ± 11.8	28.9 ± 13.1	NS
	Cellular (ng/ml)	211.0 ± 81.0	162.0 ± 81	NS
	Free/cell ratio	0.17 ± 0.07	0.2 ± 0.1	NS
+GAG	Free (ng/ml)	1073	1792	NS
	Cellular (μ g/ml)	116	54	$p < 0.02$
	Free/cell ratio	0.0092	0.033	$p < 0.05$
*Protein	Cellular (mg/ml)	10.1 ± 6.7	9.3 ± 7.5	NS

* = mean ± Standard Deviation.

+ = Geometric mean.

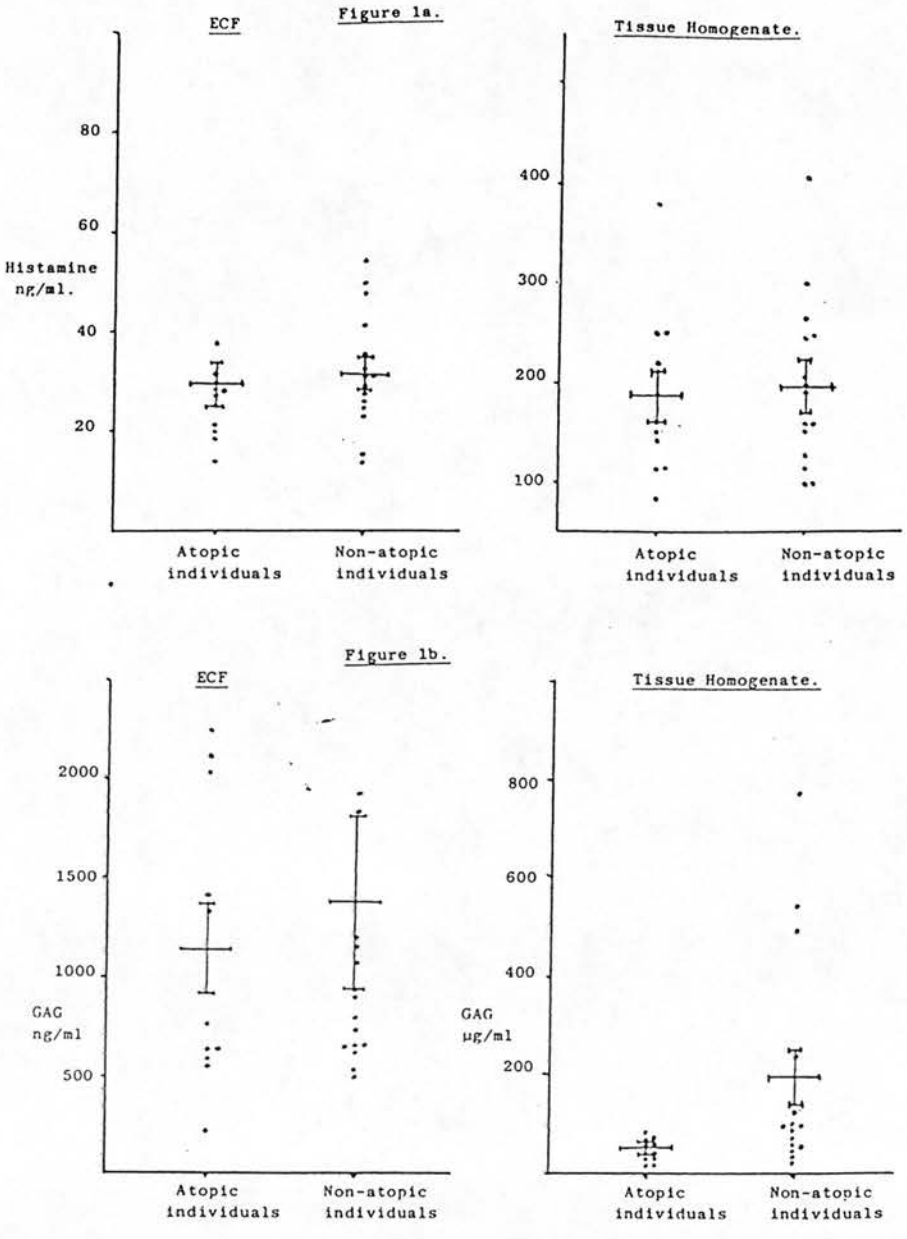


Figure 1: Concentration of Histamine and GAG in Nasal Tissue and Supernatant Fluids.

The histamine content (Figure 1a) and GAG content (Figure 1b) in tissue homogenate and extracellular fluid (ECF) samples of atopic (n = 11) and non-atopic (n = 15) individuals are shown, with mean and SEM indicated.

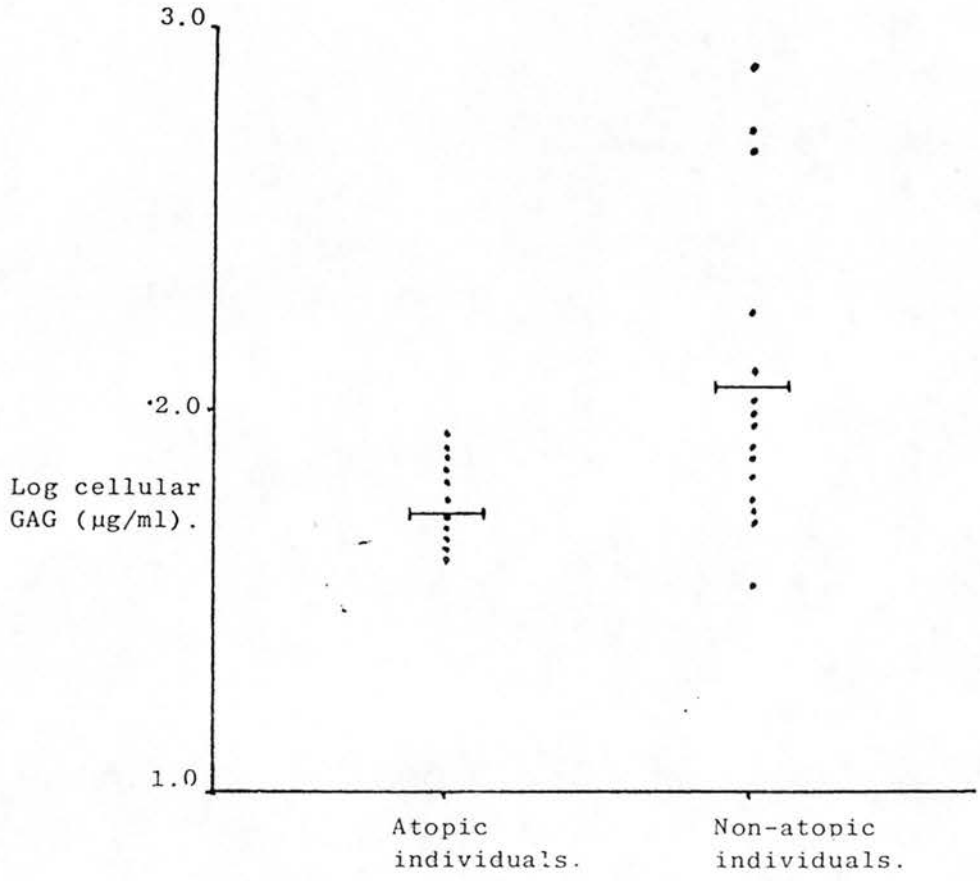


Figure 2: Log Transformed levels of Cellular GAG in Atopic and Non-Atopic Individuals.

Geometric mean is indicated.

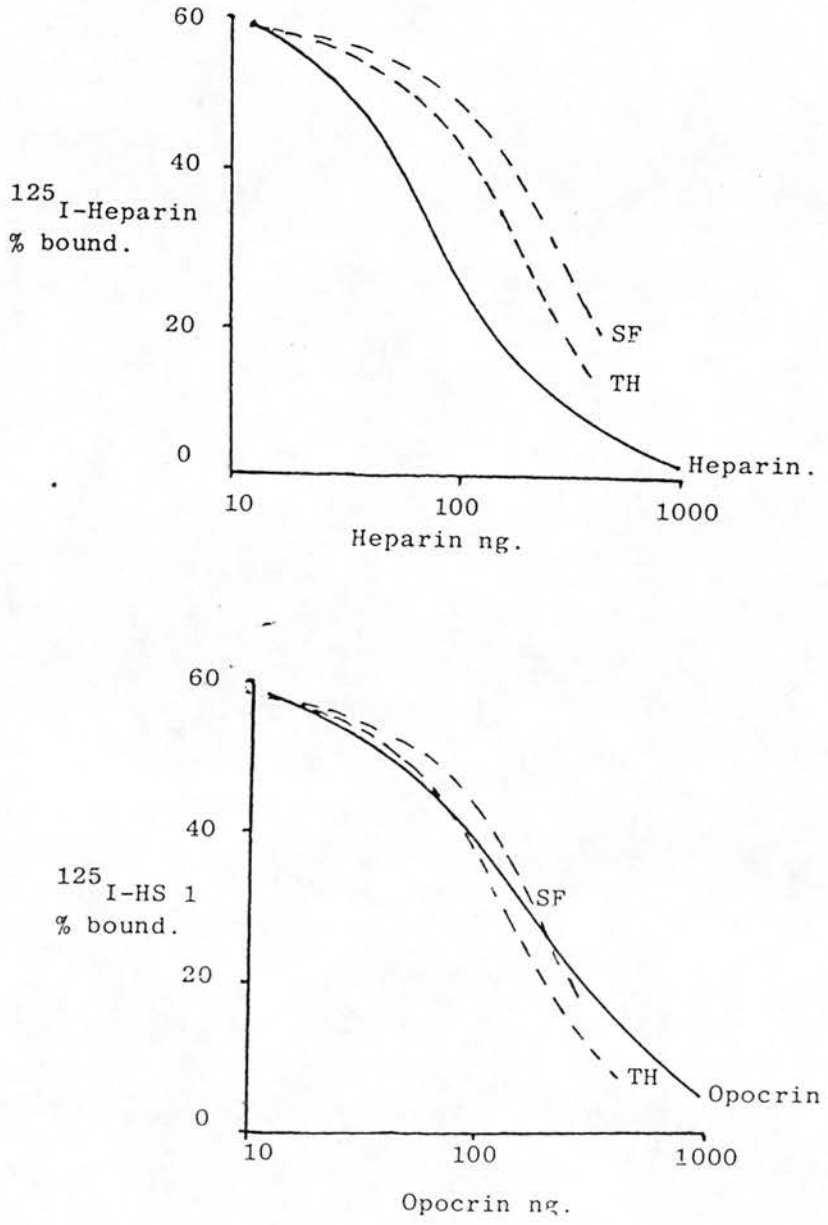


Figure 3: **Parallelism Studies of Nasal Mast Cell-Derived GAG**

Dilution curves of supernatant fluid (SF) and tissue homogenate (TH) samples in the standard GAG assay (Figure 3a), and in the heparin assay (Figure 3b).

Figure 4a.

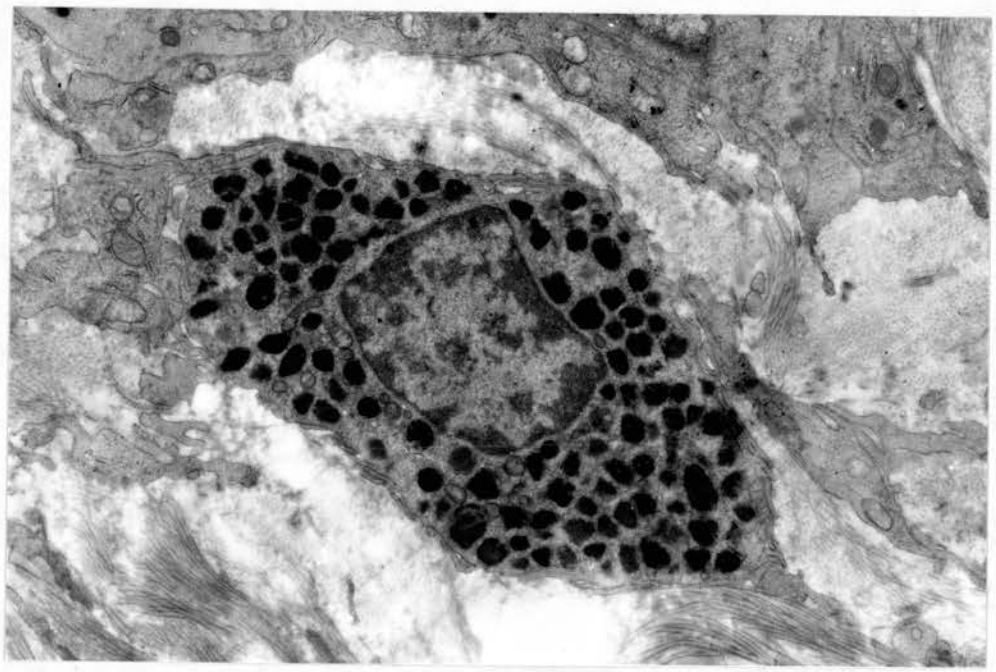


Figure 4b.

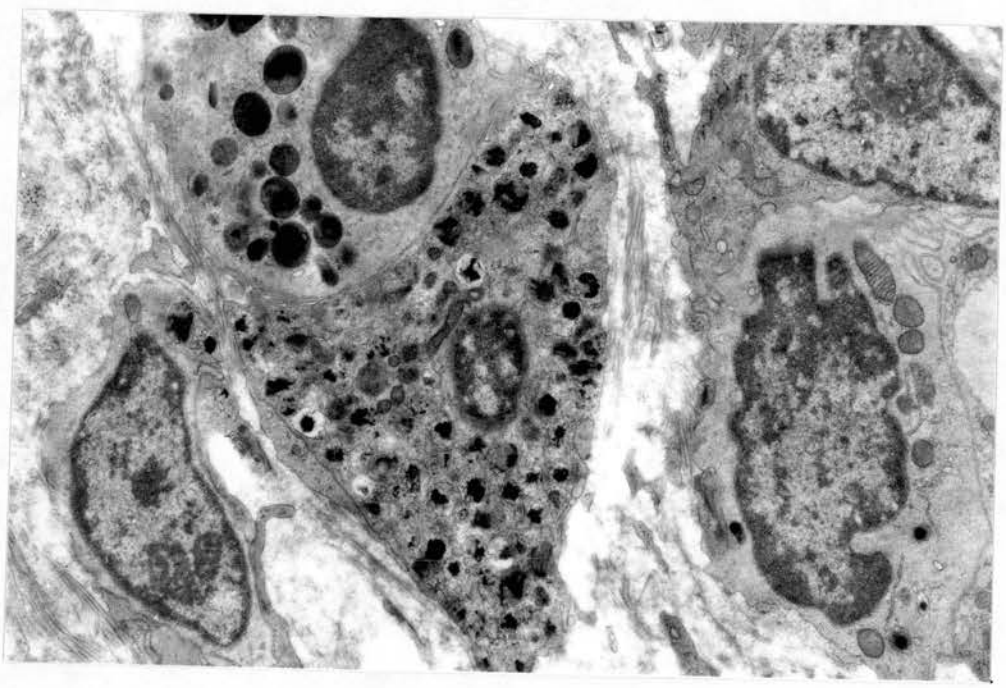


Figure 4c.

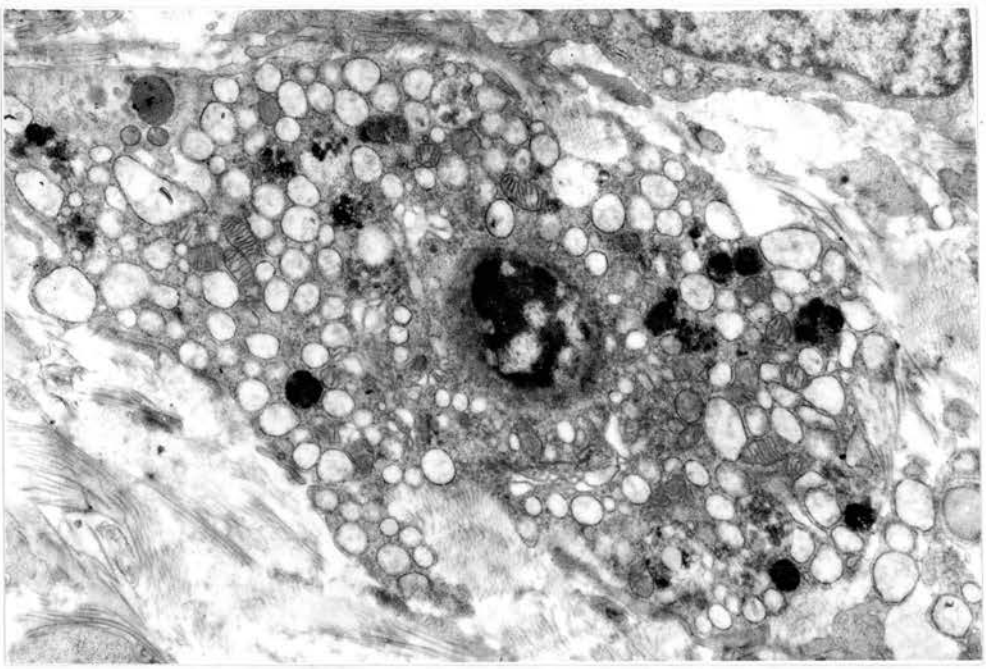


Figure 4: **Electron Micrographs of Adjacent Mast Cells in the Nasal Turbinate of an Atopic Individual.**

These electrom micrographs illustrate

a. Fully granulated mast cell.

b. Partially granulated mast cell.

c. Degranulated mast cell.

(Lead citrate and uranyl acetate, magnification= x 4200).

not correlate with total serum IgE, nor was there any association with total number of positive skin tests.

GAG Content.

Wide distributions of 'free' (210 - 20,050 ng/ml) and cellular (33 - 777 μ g/ml) GAG were observed. Mean 'free' GAG in the atopic group did not significantly differ from that of the non-atopic group, but the mean cellular GAG in the atopics was significantly less than in the non-atopics, as was the mean ratio of 'free' to cellular GAG (Table 2 and Figure 1b). There was no correlation of GAG levels with nasal histamine or serum IgE, nor any sex difference. Three non-atopic individuals had very high levels of cellular GAG (Figure 2), but no other distinctive features could be identified in these patients. As with histamine levels, the number of allergens to which each individual was sensitive did not influence the GAG levels in the atopic group.

Nature of GAG in Nasal Tissue and Supernatant Fluid.

When serial dilutions of supernatant fluid and tissue homogenate samples were assayed, the resulting curves were not parallel with the heparan sulphate standard (Figure 3a). This was then repeated using the heparin assay described in Chapter 2:1 (that is, in the presence of 50% DMSO), the material was readily assayed and produced a curve parallel with the heparin standard (Figure 3b). The GAG contained in the nasal tissue and fluid was therefore heparin-like rather than heparan sulphate-like material.

Mast Cell Counts.

The results of mast cell counting on specimens fixed in Carnoy's showed a significant difference ($p < 0.01$), between a mean of 4.4 (SD

= 5.05)/mm^2 in atopics, and 20.8 (SD = 17.2)/mm^2 in non atopics. Supplementary studies using anti-IgE or toluidine blue on 4% formaldehyde-fixed tissue did not, however, confirm the presence of a larger number of mast cells in the non-atopics. Electron micrographs of tissues indicated that mast cell degranulation is extremely variable, even among adjacent mast cells, in both the atopic and non-atopic groups. Figures 4a, 4b and 4c illustrate the varying degrees of degranulation in adjacent mast cells in the nasal tissue of an atopic individual.

(7:4). Discussion.

Allergic rhinitis affects up to 20% of the population, but as with most allergic reactions, the precise mechanisms of nasal hypersensitivity are poorly understood. Mast cells are thought to play an important role in these reactions, and to support this, increased mast cell numbers have been detected in nasal smears from patients with rhinitis (Mygind and Thomsen 1973). Furthermore, nasal provocation tests with pharmacological agents such as histamine and/or metacholine induce the symptoms of allergic rhinitis, sneezing and marked vasodilation probably via both H_1 and H_2 receptors in the nasal mucosa (Secher et al 1982). Nasal provocation tests which administer antigen to the nose thereby causing mediator release, are also indicative of allergic rhinitis. The concentration of histamine in nasal fluids has previously been shown to be extremely variable, and unrelated to an individual's atopic status (Drake-Lee and McLaughlan 1982; Drake-Lee et al 1984; Drake-Lee 1984). Histamine levels in normal nasal mucosa varied over a similar range to that reported in nasal polyps. In this

study however a variable biological assay was used for the detection of histamine in small turbinate biopsies (Baxter and Rose 1953), and subsequent studies have relied on the collection of nasal washings or epithelial curettings and have produced conflicting results (Hamaguchi et al 1983; Eggleston et al 1978; Naclerio et al 1983; Otsuka and Okuda 1981). A study of the release of mast cell granule-derived GAG in human nasal tissue has not previously been reported.

The results obtained from the 26 patients examined confirmed the previous reports of variable histamine levels in fluids and tissues of the nose. The results indicate that in chronic rhinitis there are significant basal levels of 'free' histamine (range 14-65 ng/ml), the corresponding plasma value being < 10 ng/ml. The histamine levels in the tissue homogenate samples were much greater, with a range of 81-405 ng/ml. There were no significant differences in either 'free' or residual cellular histamine levels between the atopic and non-atopic groups, perhaps due to the masking of differences by the rapid metabolism of released histamine by histaminase (Schwartz and Austen 1984) outside the secretory granule. Any release of histamine from the mast cells into supernatant fluid would therefore not be detected, due to its very short half life.

The GAG contained in, and released from the human mast cells studied resembled heparin rather than the heparan sulphate-like GAG in the basophils. Parallelism studies using doubling dilutions of both supernatant fluid and tissue homogenate samples in the GAG assay revealed that the material contained in these samples was more sulphated than the heparan sulphate standard (Figure 3a). Subsequent analysis of these samples using the assay specific for

heparin produced dilution curves parallel with the heparin standard in both supernatant fluids and tissue homogenates (Figure 3b). The involvement of basophils in allergic rhinitis has previously been reported (Hastie et al 1979; Okuda et al 1978; Okuda and Otsuka 1977), and circulating basophils are thought to migrate to the nasal mucosal surface (Okuda et al 1979). Any basophil-derived GAG contained in these samples would not be detected in the heparin assay. Nevertheless, basophils have previously been shown (Chapter 3) to contain no heparin-like material. As no source of heparin other than mast cells has been identified, it may therefore be concluded that the material detected in supernatant fluid samples originated from the mast cells of the nasal mucosa.

A wide range of 'free' GAG levels was also detected in both atopic and non-atopic groups, but these did not correlate with 'free' histamine levels. This is consistent with the findings of Chapter 6, whereby the release of GAG and histamine from basophils appear to be dissociated and vary widely in both atopic and non-atopic individuals. Overall, 'free' GAG levels were greater in the atopic group, but this difference failed to reach statistical significance. However, residual cellular GAG levels were significantly greater in the non-atopic individuals; the mast cell counts on Carnoy's fixed specimens suggested that this might simply reflect a larger number of granulated mast cells in the tissue of non-atopic individuals. However, further counts using different staining procedures on 4% neutral buffered formaldehyde-fixed specimens gave nasal mast cell numbers in atopic individuals similar to or slightly greater than those of non-atopics. This may indicate the possible existence of different subpopulations of mast cells within nasal tissues, which are selectively demonstrated by

the methods used. A recent report by Shanahan et al (1987) showed that histochemically distinct mast cell subpopulations which can be identified by different fixation and staining procedures exist in human lung tissue. The findings presented here suggest that a similar heterogeneity may also exist in mast cells of the nasal mucosa. Furthermore, functional heterogeneity of human mast cells from different anatomical sites in vivo has recently been reported (Tharp et al 1987). It is possible that GAG in mast cells of atopic individuals exists in a more 'releasable' form than in those of non-atopic individuals, and therefore cellular stores of GAG in atopics may have been depleted prior to surgery. Alternatively, cellular stores of GAG in atopic individuals may have been depleted due to allergenic stimulation. Nasal mucosa of non-atopic individuals appeared to contain a higher proportion of granulated mast cells demonstrated by Carnoy's fixation which may contain excess stores of less 'releasable' or 'non-releasable' GAGs. These data are consistent with those obtained from the studies on basophils (Chapter 6), and collectively point to the possible existence of more than one storage pool for secretory granule mediators in both mast cells and basophils.

Chapter 8.

Plasma GAG Levels in Health and Disease.

(8:1). Introduction.

The studies outlined in this thesis so far have indicated that GAGs found in human plasma have similar biochemical characteristics to those contained in the circulating basophils. The highly sulphated GAGs contained in human tissue mast cells do not appear in plasma at detectable levels. Plasma GAG may therefore, at least in part, be basophil-derived (Chapter 3). The studies outlined in this Chapter have examined further the relationship between plasma GAG and circulating basophils and its physiological importance in the pathogenesis of type 1 hypersensitivity reactions.

In order to assess plasma GAG levels in health and disease, circulating levels of plasma GAG were measured in atopic and non-atopic individuals and related to their individual basophil counts. Sequential blood samples were also taken from a number of both atopic and non-atopic individuals, to detect variations of basophil counts with time. In addition, a group of patients with various related myeloproliferative diseases including chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL), acute myeloblastic leukaemia (AML), and acute lymphoblastic leukaemia (ALL), in which circulating basophils (Parwaresch 1976) and histamine (Jacquiliat 1975) are known to be elevated, were studied to provide a population for comparison.

Plasma GAG levels were measured in a group of patients prone to anaphylactic and anaphylactoid reactions, which are particularly difficult to study in humans, due to their rapid nature and serious

consequences. Severe systemic reactions of this kind are occasionally experienced by patients under certain clinical conditions where they can be closely monitored: such as following the transfusion of blood or blood products, including intravenous immunoglobulin. Adverse reactions associated with the administration of intravenous immunoglobulin have been attributed to a number of factors, including the presence of aggregates of IgG in the preparations (Barundun et al 1962; Barundun et al 1979), vasoactive peptides (Alving et al 1980), endotoxins (Hanson et al 1979), anti IgA antibodies (Vyas et al 1969; Burks et al 1986), and the physiological effects of the immunoglobulin (Barundun et al 1962). A decrease in the proportion of aggregated immunoglobulin in the products available from various manufacturers following changes in preparative procedures, has been associated with a decrease in the incidence of adverse reactions (Eibl et al 1984; Pirofsky 1984). However, the precise mechanisms of these adverse reactions remain poorly understood.

The possibility of basophil involvement in these reactions, as measured by plasma GAG and histamine, was therefore assessed by studying:

a. a group of 15 patients before and after platelet transfusion.
b. a group of 18 patients with primary disorders of immunoglobulin production, during a blind crossover evaluation of a new preparation of immunoglobulin for intravenous use.

(8:2). Patients, Materials and Methods.

Patients.

Control Individuals.

35 healthy adult volunteers, described in Chapter 5, were non-atopic controls.

Atopic Individuals.

55 atopic individuals, described in Chapter 5, were studied in total.

Leukaemia Patients.

A total of 34 patients were studied at various stages of therapy; 17 patients aged 20 - 58 years (mean age = 45.7 years) had Philadelphia chromosome positive CML in chronic phase, 9 aged 49 - 82 years (mean age = 63.9 years) had CLL, 5 aged 39 - 45 years (mean age 42.3 years) had AML, and 3 aged 48 - 67 (mean age = 55.3 years) had ALL.

Patients Receiving Blood and Blood Products.

Platelet Transfusions.

Plasma samples were collected from 15 patients receiving platelet transfusions.

Intravenous Immunoglobulin Preparations.

A total of 18 patients (15 male; 3 female) aged 18 months to 20 years (median age = 10 years 10 months), who were attending an immunoglobulin replacement clinic of the Royal Childrens Hospital, Melbourne, Australia, were studied. All had been receiving intravenous immunoglobulin replacement therapy every four weeks at a dose of 300 to 450 mg/kg per infusion. The median duration of

intravenous therapy prior to enrolment was 3 to 5 years (range = 1 month to 10 years). None of the patients had acute infections at the time of study. Ten patients had experienced frequent adverse reactions to the intravenous immunoglobulin preparation used as standard therapy, frequent reactions being defined as infusion-related symptoms necessitating slowing or interruption of the infusion on at least three occasions during the six month period preceding the study. This study was performed in collaboration with Dr DM Robertson and Dr CS Hosking, who carried out the clinical parts of the study, and collected the samples for assay.

Materials.

Intravenous Immunoglobulin Preparations.

The two intravenous immunoglobulin preparations were prepared by the Commonwealth Serum Laboratories (CSL), Parkville, Australia, and were supplied as 6% (w/v) solutions. Both were prepared from pooled volunteer blood donor plasma by ethanol fractionation. "Standard" CSL normal human immunoglobulin for intravenous use was briefly exposed to pH 4.0, followed by adjustment to pH 6.4. The preparation did not contain maltose and had been used continuously for previous therapy for all 18 patients. A modified preparation ("low pH" immunoglobulin for intravenous use) was maintained at pH 4.0 and contained 10% maltose.

The "standard" CSL immnnunoglobulin preparation for intravenous use contained approximately 18% aggregated IgG, whereas the aggregate content of the low pH product was less than 5%.

Methods.

Collection of Samples.

Whole blood samples were taken into trisodium citrate (3.12% w/v), and plasma obtained as described in Chapter 2:1.

Basophil Counts.

Whole blood samples were collected into EDTA (0.025M) vacutainer tubes and cell counts determined as described in Chapter 5.

Assay of GAG

Samples were prepared and assayed for GAG by the method described in Chapter 2:1.

Assay of Histamine.

Samples were prepared and assayed for histamine by the method decribed in Chapter 2:2.

Sequential Studies of Plasma GAG.

Daily Variation of Plasma GAG.

Duplicate plasma samples were obtained from 2 atopic and 2 non-atopic individuals at the same time on 5 consecutive days, and assayed for GAG.

Diurnal Variation of Plasma GAG.

Plasma samples were prepared in duplicate from 5 atopic and 3 non-atopic individuals at intervals of 3h over a period of 24h, and assayed for GAG.

Platelet Transfusions.

Three samples were collected from each patient; one just before the

transfusion, and two further samples, 1h and 24h after the transfusion. Patients were closely monitored for any adverse reaction to the platelet transfusion.

Intravenous Immunoglobulin.

This study was a blind crossover trial, with each patient receiving one intravenous infusion of the standard immunoglobulin and one infusion of the low pH immunoglobulin. Nine patient pairs were assigned by random allocation, with one patient in each pair receiving the standard product and one the low pH product at the initial infusion. The order was reversed for each patient pair for the second infusion given four weeks later. No medications were given during the 48h prior to each infusion.

All infusions were given at the same rate of 0.75ml/min/20kg patient body weight for the first 30min, and subsequently at 1.5ml/min/20kg patient body weight. Each patient received their usual dose of immunoglobulin and the same volume of immunoglobulin was prescribed for each treatment. Samples were taken immediately prior to each infusion and 1h after commencement of the infusion. Further samples were taken at 2h and 3h if the infusion had not been completed at these times. Samples were collected by aspiration through intravenous cannula, also used for administration. Samples were stored at -70°C, coded prior to testing, and assayed without knowledge of the ocurrence of reactions or the type of immunoglobulin received.

Statistical Analysis.

Significance tests on all plasma GAG levels were analysed by Student's t-test on log transformed data, except for the

intravenous immunoglobulin study which used the McNemar test for significance of changes with correction for continuity, the Wilcoxon matched pairs signed ranks test and the Fisher exact test.

(8:3). Results.

Plasma GAG Levels.

Atopic and Non-atopic Individuals.

Levels of plasma GAG did not differ significantly between atopic (range = 210-905; mean = 432.2; SEM = 29.8 ng/ml) and non-atopic (range = 205-1005; mean = 418.1; SEM= 28.2 ng/ml) groups ($p = 0.32$), (Figure 1). Plasma GAG did not correlate with basophil counts in either atopic ($r = 0.09$) or non-atopic ($r = 0.19$) groups.

Leukaemia Patients.

Plasma GAG was significantly raised above normal in patients with CML (range = 615-5,350; mean = 1993.5; SEM = 303.3 ng/ml), ($p< 0.001$), (Figure 2); circulating basophil counts (range = 20-5,480; mean = 1405.3; SEM = 403 x 10^6/l), (Figure 3), and eosinophil counts (range = 60-4,380; mean = 919.4; SEM = 240.4 x 10^6/l), (Figure 4) were also raised in these patients. Moreover, basophil and eosinophil counts correlated in this group ($p< 0.005$). A strong correlation ($r = 0.80$, $p< 0.001$; regression equation $y = 2.06 + 0.35 x$) was observed between plasma GAG and absolute basophil counts in the group of leukaemic patients as a whole, suggesting that increased GAG levels in plasma can directly reflect elevated absolute basophil numbers.

Daily Variation of Plasma GAG.

Plasma GAG levels fluctuated considerably from day to day, with no

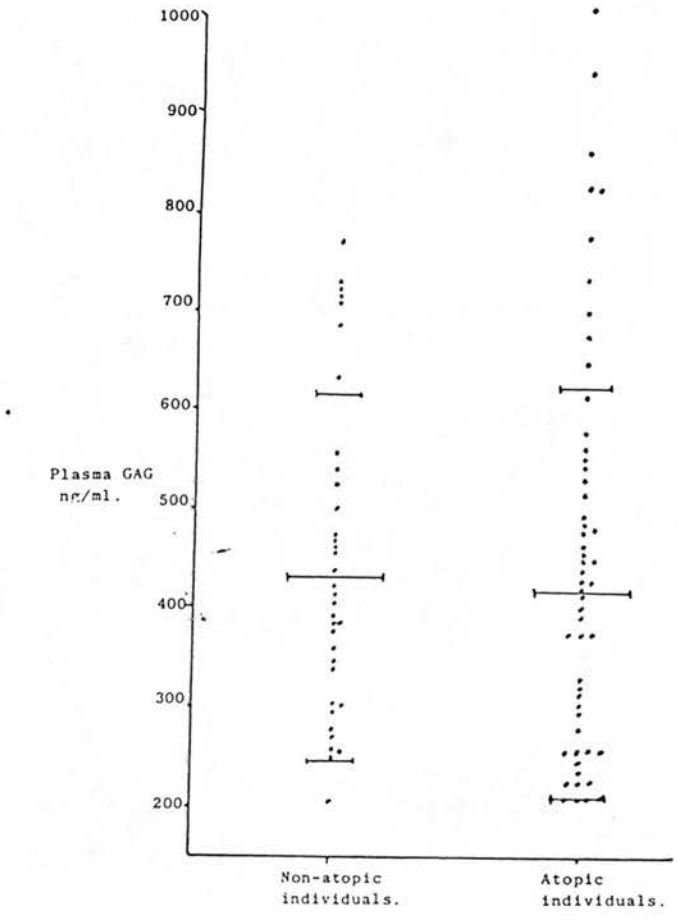


Figure 1: **Plasma GAG Content of Atopic and Non-Atopic Individuals.**

Levels of plasma GAG did not significantly differ between atopic and non-atopic groups ($p = 0.32$). Mean values ± SD are indicated.

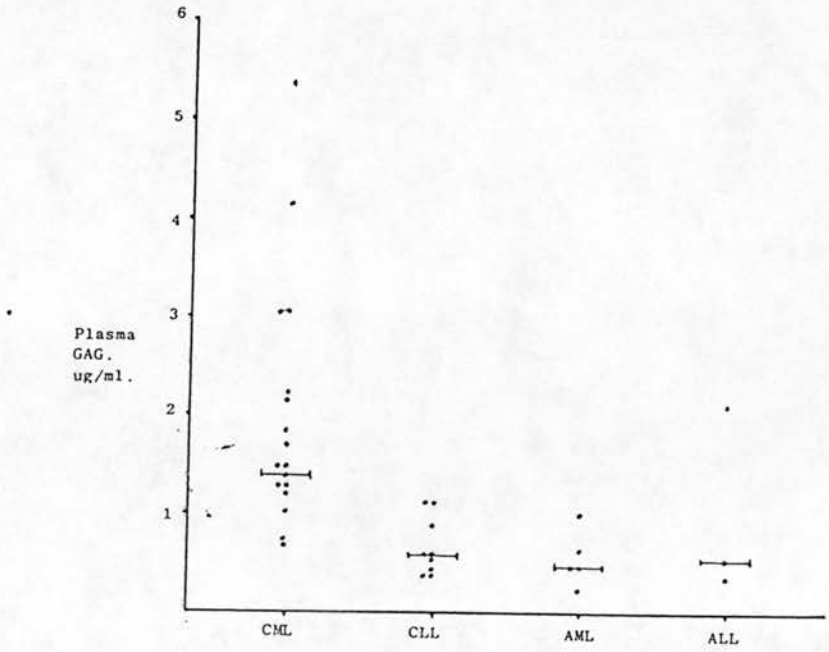


Figure 2: **Plasma GAG Content of Patients with Various Related Myeloproliferative Disorders.**

Plasma GAG was significantly raised above normal in patients with CML ($p < 0.001$). Mean values are indicated.

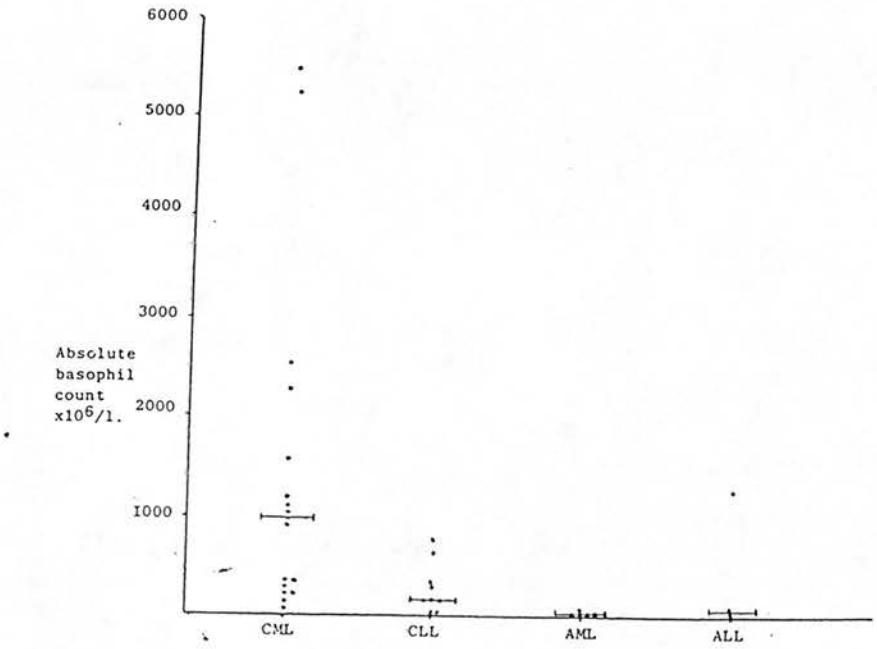


Figure 3: **Circulating Basophil Counts in Patients with Various Related Myeloproliferative Disorders.**

Circulating basophil counts were significantly raised above normal in patients with CML ($p < 0.005$). Mean values are indicated.

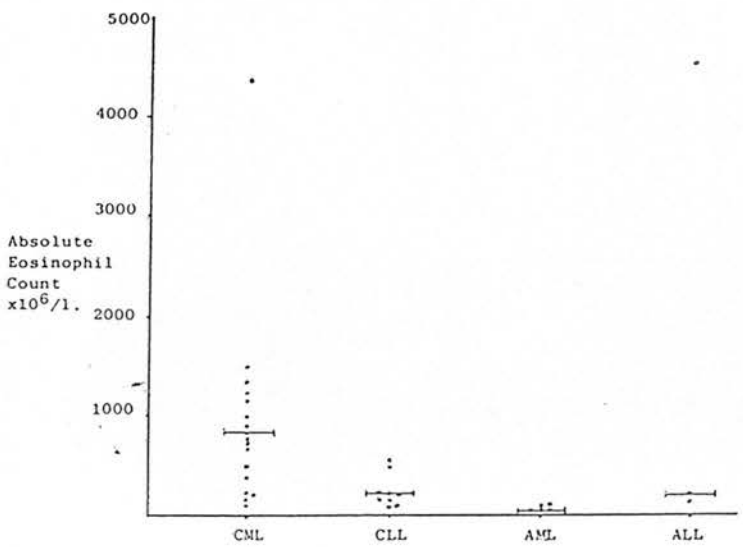


Figure 4: **Circulating Eosinophil Counts in Patients with Various Related Myeloproliferative Disorders.**

Eosinophil counts were significantly raised above normal in patients with CML ($p < 0.005$). Mean values are indicated.

obvious pattern and with no apparent differences between the atopic and non-atopic individuals (Figure 5). There was no correlation between the variation of plasma GAG and basophil counts in either group.

Diurnal Variation.

A wide variation of plasma GAG levels occurred both within and between each population studied (Figures 6a and 6b). Again no obvious pattern of fluctuation was observed.

Plasma GAG Levels in Patients Receiving Blood and Blood Products.

Platelet Transfusions.

There was no obvious pattern of fluctuating plasma GAG levels in any of the patients studied during the course of uneventful platelet transfusions (Figure 7); none of the patients exhibited any adverse reactions during treatment.

Intravenous Immunoglobulin Study.

Plasma Histamine Content.

Plasma histamine concentrations in all samples taken before and during infusion with either immunoglobulin preparation were below the lower limit of detection of the assay, which was 5 ng/ml.

Plasma GAG Content.

The median plasma GAG contents prior to and during infusions are shown in Table 1. There were no significant changes in GAG concentrations during treatment with either type of immunoglobulin, nor were there any significant differences between the two treatment groups at any of the sampling times (Wilcoxon matched pairs signed ranks tests).

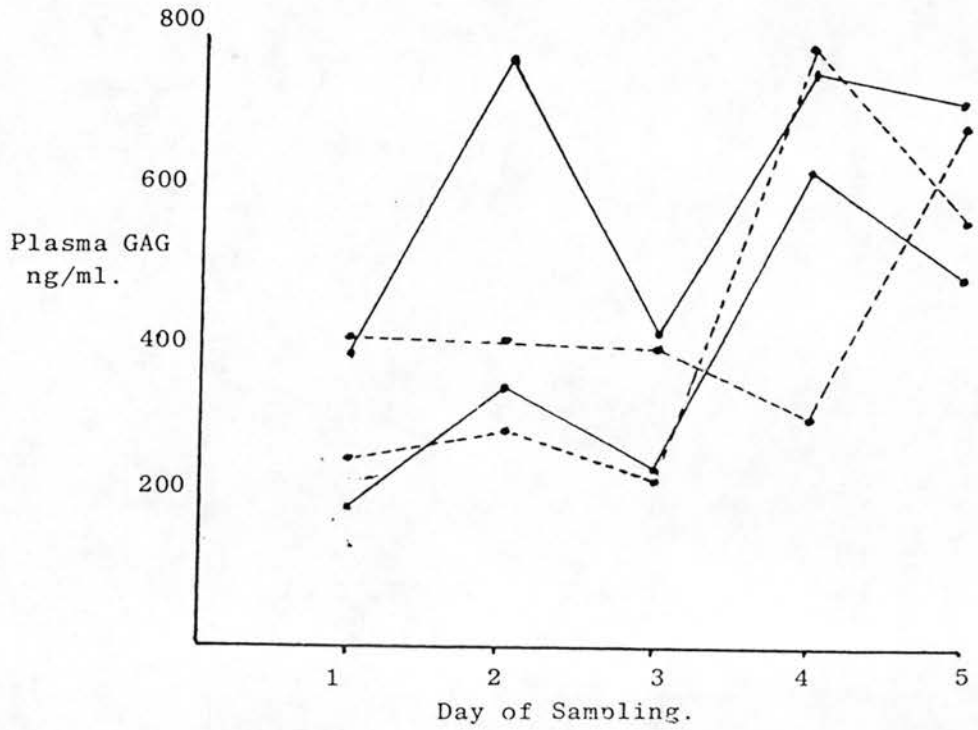


Figure 5: **Daily Fluctuations of Plasma GAG Concentrations.**

Plasma GAG levels fluctuated considerably from day too day in both atopic (---), and non-atopic (——) groups.

Figure 6a.

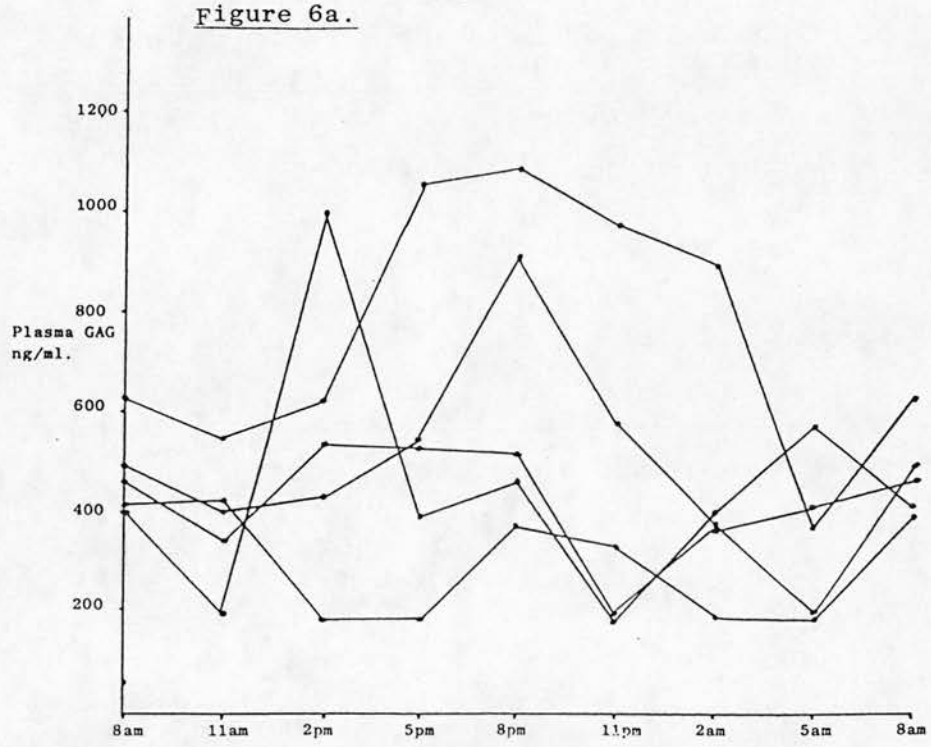


Figure 6b.

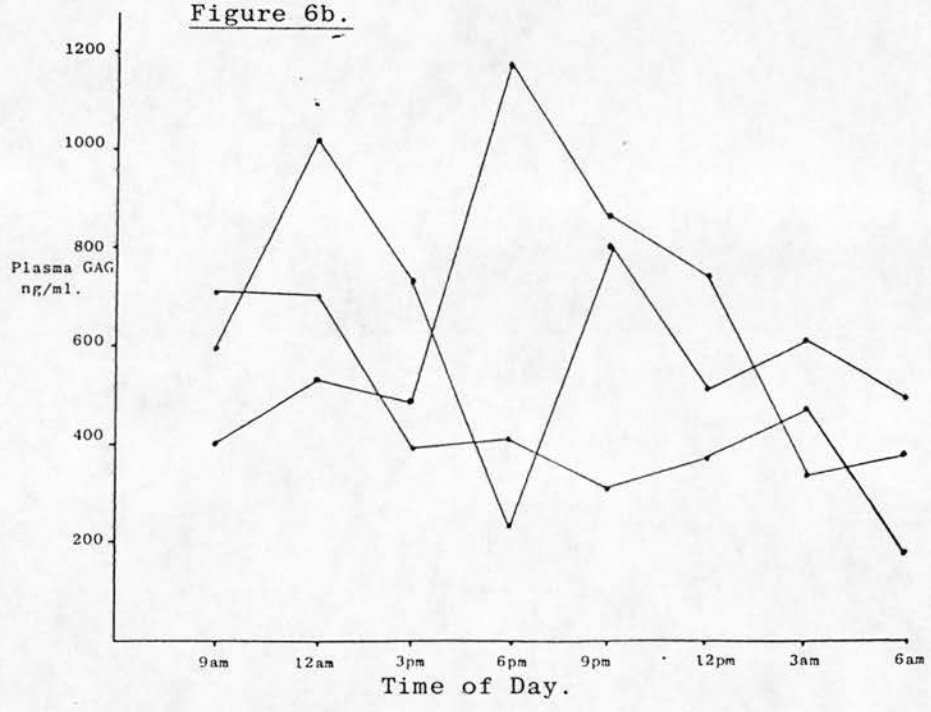


Figure 6: **Diurnal Variation of Plasma GAG Concentration.**

A wide variation of plasma GAG levels occurred both within and between atopic (Figure 6a), and non-atopic (Figure 6b) groups, with no obvious pattern of fluctuation.

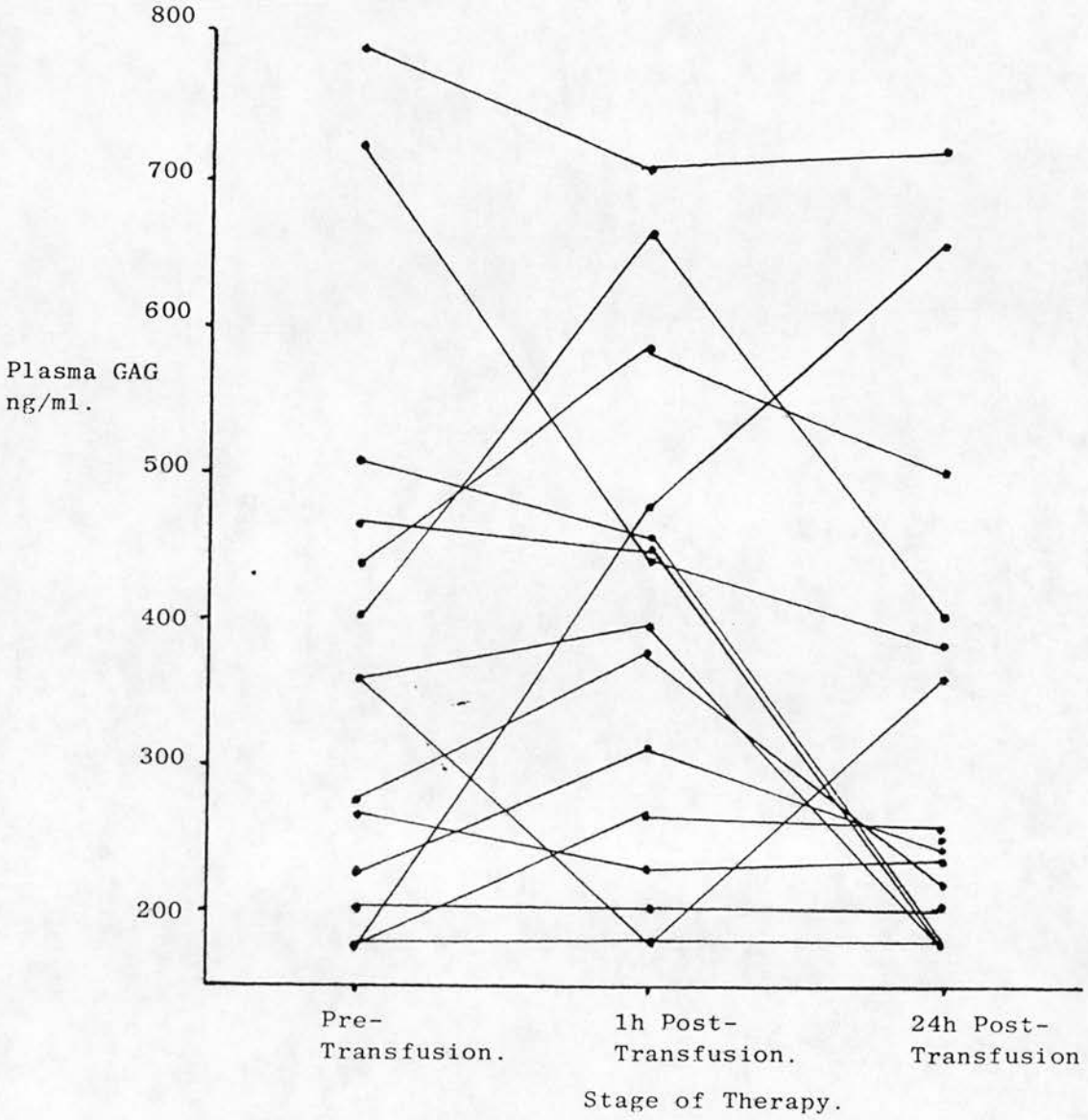


Figure 7: Plasma GAG concentrations of Patients at Various Stages of Platelet Transfusion.

No obvious pattern of fluctuation of plasma GAG levels occurred durin the course of transfusion; none of the patients exhibited any adverse reactions during treatment.

	Plasma GAG (ng/ml).			
	Pre-infusion.	1hour.	2hours.	3hours.
Standard Immunoglobulin.	890	985	805	755
(range)	< 200-3800	< 200-4800	385-14,600	500-4000
Low pH Immunoglobulin	1290	775	1020	-
(range)	< 200-3950	< 200-2100	< 200-2420	-

Table 1. **Plasma GAG levels before and during infusion with standard (aggregate-containing) and low pH immunoglobulin preparations.**

Values shown are medians. No samples were taken at 3 hours in the low molecular weight immunoglobulin group, since all infusions were completed by this time.

(8:4). Discussion.

The glycosaminoglycans circulating in human plasma have been shown in previous Chapters to be indistinguishable from those contained in human basophils (Chapter 2:1 and Chapter 3). Moreover, this GAG has been found to be exclusively contained in the basophil fraction of whole blood (Chapter 3), and plasma GAG may therefore originate at least in part from circulating basophils; the relationship between plasma GAG and individual basophil counts in various groups was therefore studied. Plasma GAG levels varied considerably within atopic and non-atopic groups, and unlike basophil counts (Chapter 5) plasma GAG did not differ significantly between these two populations (Figure 1). Basophil counts were considerably raised above normal in many of the leukaemic patients studied, and previous reports that the greatest elevations of basophils occur in CML (Forkner 1938; Doan and Reinhart 1941; James et al 1955; Juhlin 1963b) were confirmed (Figure 3). The increasing basophilia associated with CML may indicate the development of blast transformation (Edzinli et al 1970; Theologides 1972; Jacquillat et al 1975), and these basophils do show a number of inherent biochemical and functional defects (Lett-Brown and Juneja 1985). The raised eosinophil counts in these individuals (Figure 4) are consistent with the findings of Chapter 5, in which circulating basophil and eosinophil levels of both atopic and non-atopic individuals are closely correlated. Histamine levels are known to be elevated in CML (Jacquiliat 1975), and plasma GAG levels were also found to be significantly elevated in this group (Figure 2). At these concentrations, plasma GAG was closely correlated with the absolute basophil count ($r = 0.08$, $p < 0.001$; regression equation $y = 2.06 + 0.35x$). Such a close relationship would suggest that the

increased plasma GAG levels directly reflected elevated absolute numbers of basophils, rather than GAG release from another cell, or immunologically mediated basophil activation with granule release, for which no obvious precipitating factors were observed. Although the levels of plasma GAG detected were much lower than levels observed in patients with GAG-associated bleeding tendencies (Dawes, personal communication), sequential plasma GAG measurements on CML patients to monitor any increase during the accelerated phase, with complementary coagulation studies to determine any physiological effect may be of interest in patients with untreated CML. Although basophil counts were higher in atopic than non-atopic groups, differences were atopic groups, differences were not sufficiently marked to influence GAG levels in plasma.

The levels of plasma GAG in both atopic and non-atopic individuals fluctuated with time (Figures 5 and 6); no obvious pattern of variation or external factors responsible for such fluctuations could be identified in either group. This probably reflects release of granule-associated GAG from basophils and its metabolism *in vivo*.

None of the patients receiving platelet transfusions experienced adverse reactions. Plasma GAG levels did fluctuate pre- and post-transfusion in many cases (Figure 7), but were not apparently influenced by the treatment administered; rather, they probably reflect the variation described above (Figures 5 and 6).

By contrast, adverse reactions were frequently associated with the infusion of intravenous immunoglobulin. In the blind crossover study described, a strong association was shown between adverse reactions during the infusion, and the presence of high molecular weight IgG in the immunoglobulin preparation (Robertson *et al*;

submitted for publication). Plasma histamine and GAG levels did not change significantly during infusions with either immunoglobulin preparation, or with the occurrence of adverse reactions during infusion. Although the apparent lack of histamine release could simply be due to the rapid metabolism of any released histamine by histaminase in plasma, it is consistent with previous reports that antihistamines and sodium chromoglycate are not useful as treatment or prophylaxis of adverse reactions (Pirofsky 1984). Moreover, the failure to detect any significant change in plasma GAG also appears to indicate that basophil release is unlikely to occur during these reactions. In this study, it was shown that although conversion of C_3 occurred in plasma from patients receiving the aggregate-containing preparations, there was no quantitative correlation with either the incidence or severity of reactions. Plasma granulocyte elastase concentrations also increased during infusion of the aggregate-containing immunoglobulin, and patients experiencing reactions tended to have higher plasma granulocyte elastase concentrations. This was not however statistically significant, and was independent of the degree of C_3 conversion. It may reflect in vivo stimulation of polymorphonuclear leucocytes (PMN), by the binding of aggregated IgG to F_c receptors on neutrophils. None of the parameters measured were indicative of incidence or severity of reactions experienced by patients.

The finding that hypogammaglobulinaemic patients capable of producing some immunoglobulin are more likely to experience adverse reactions suggests that investigations directed towards the detection of anti-isotypic antibody, or even perhaps idiotype-anti-idiotype reactions (Cunningham-Rundles 1982), may be more useful in further elucidating the mechanisms of adverse reactions associated with intravenous IgG administration.

Chapter 9.

General Discussion.

Release of histamine from basophils has served as an excellent *in vitro* assay of immediate hypersensitivity for many years, but knowledge of the actual participation of these cells in such reactions has to date been limited. The measurement of GAGs has added to the existing knowledge of human basophils in both health and disease.

The major findings of the studies outlined in this thesis are summarized as follows:

a. Highly sulphated GAGs were detected in plasma and the basophil fraction of human whole blood. The nature of plasma GAG was indistinguishable from that of basophil-derived GAG. Plasma GAG may therefore originate at least in part from circulating basophils. Approximately 70% of the highly sulphated GAGs in basophils was heparan sulphate and 30% was highly sulphated chondroitin sulphate, probably chondroitin sulphate E.

b. These GAGs are released from basophils of atopic individuals following Ca^{2+}- and dose-dependent allergenic stimulation with *D. pteronyssinus*. There was also some release from basophils of non-atopic individuals, which probably occurred by a non-immunological mechanism. Allergen-induced release of histamine occurred only from basophils of atopic individuals, and differed markedly from the release of GAG both in time course, and allergen concentration required for optimum release.

c. The major GAGs contained in rat mucosal mast cells and cultured rat basophilic leukaemia cells had similar biochemical properties

to those of human basophils: these were released together with histamine and RMCP II protease (both also mucosal mast cell-derived) during systemic anaphylaxis of Nippostrongylus brasiliensis-primed rats challenged intravenously with worm antigen.

d. Circulating basophil numbers in atopic individuals were significantly elevated above those of non-atopic individuals, as were eosinophil counts, which paralleled those of basophils in both populations. Individual basophil counts did not fluctuate over short or long periods of time in either atopic or non-atopic individuals.

e. GAG contained in mast cells of nasal mucosa was heparin, and was present in significantly greater concentrations in non-atopic than atopic individuals.

f. Plasma GAG levels did not differ significantly between atopic and non-atopic populations, but individual GAG levels fluctuated considerably. However, in a group of patients with CML with very high basophil counts, plasma GAG levels were raised and there was significant correlation of plasma GAG and circulating basophil numbers.

g. There was no significant increase in plasma GAG levels in patients following uneventful platelet transfusions. No significant increase of plasma GAG or histamine levels was seen in patients receiving intravenous immunoglobulin, although raised levels of granulocyte elastase and complement breakdown products were observed in many patients experiencing adverse reactions. This was a good indication that basophils were not involved in the mechanisms of adverse reactions to infusions of intravenous immunoglobulin preparations.

The release of GAG but not histamine from the secretory granules of basophils from non-atopic individuals after exposure to a D. pteronyssinus preparation raises the possibility that more than one type of storage pool exists for different mediators in these cells. This is supported by the findings from the studies of nasal tissue mast cells. Cellular stores of GAG were present in significantly greater concentrations in non-atopic individuals than in atopic individuals, whereas cell numbers and both cellular and extracellular histamine concentrations remained constant in both populations.

The degranulation of basophils during type 1 hypersensitivity reactions involves the crosslinking of adjacent IgE receptors on the cell surface, and results in the release of both stored and newly synthesized mediators from the cytoplasmic secretory granules. Since histamine is closely associated with GAGs in these organelles, it has generally been assumed that they will be released together, and by the same mechanism, during degranulation. Evidence presented here points to the existence of a complex process, in which release of GAG and histamine occurs at different rates, and possibly by different mechanisms.

It has been confirmed that human nasal tissue mast cell GAG is largely heparin, whereas basophils contain the less sulphated GAGs also present in plasma. Previous studies in patients have defined these GAGs as chondroitin sulphates, but evidence presented here suggests that of the highly sulphated GAGs in normal human basophils, approximately 70% was heparan sulphate and 30% was highly sulphated chondroitin sulphate, probably chondroitin sulphate E.

The release of mediators from mast cells and basophils appears to

play a central role in immediate hypersensitivity reactions, and clearly all of these mediators could have clinical importance in the manifestations of allergic diseases. However, the exact role of the secretory granule-derived GAG, when released, is unclear. The most obvious physiological function (other than packaging of the granule mediators) for mast cell-derived heparin is as an anticoagulant at local sites of inflammation. In addition, heparin has many other functions described in Chapter (2:1); these include the inhibition of cell migration and growth, binding to and altering the activity of eosinophil-derived major basic protein (Gleich 1977), and possibly inhibiting complement activation at several steps along the cascade (Weiler et al 1970). Basophils do not contain heparin, but rather possess less sulphated proteoglycans, heparan sulphate and 'oversulphated' chondroitin sulphate E. Heparan sulphate does have anticoagulant activity, though approximately twenty times less than that of heparin, and the anticoagulant activity of chondroitin sulphate E has not yet been determined. The anticoagulant effect of GAGs released from basophils may be less important than that of mast cells, since basophils are not so directly involved at local sites of inflammation in type 1 hypersensitivity reactions. Furthermore, if basophil-derived proteoglycan had anticoagulant activity as potent as that of heparin, general systemic coagulation problems could result in many individuals.

The involvement of mast cells and basophils in human anaphylaxis is thought to be of considerable importance, but direct evidence of their role in such reactions is difficult to obtain. The importance of basophils in type 1 hypersensitivity reactions has been highlighted by the finding that basophils were significantly

elevated above normal in a large group of atopic individuals. With the use of the Nippostrongylus-primed rat model, the mechanisms of anaphylaxis could be studied in greater detail. Knowledge of mast cell involvement during anaphylaxis from studies on rat mucosal mast cell-derived RMCP II, has been supplemented with histamine and GAG measurement at the same time and from the same cells. From these studies, it was clear that generalized systemic release of mediators occurs during anaphylaxis. It was also confirmed that unlike connective tissue mast cells, rodent mucosal mast cells did not contain heparin, but less sulphated GAGs of similar biochemical nature to those contained in human basophils, contributing to the evidence for mast cell heterogeneity.

The measurement of highly sulphated GAG and its release during the degranulation process has proved valuable in the study of normal human basophils and their involvement in type 1 anaphylactic hypersensitivity reactions. Although the exact role of the basophil in allergic disease and type 1 hypersensitivity reactions remains unclear, the information presented in this thesis underlines that it is likely to be of considerable importance.

Future Investigations.

Biochemical Studies.

One of the major findings of this study was the characterization of the highly sulphated GAGs derived from human basophils as heparan sulphate and oversulphated chondroitin sulphate. However, these experiments used extracts of GAG from relatively crude basophil-enriched fractions of whole blood, and should be repeated using more highly purified GAG extracts. GAGs from a basophil-enriched fraction obtained by density gradient centrifugation as described in Chapter 3 should initially be purified on Polybrene-Sepharose and then subjected to specific enzymic and chemical degradation procedures. Finally, identification of the resulting disaccharides would permit clear delineation of the GAG structure.

The GAG content and composition of human basophils must be studied further in a physiological context, to determine whether the ratio of heparan sulphate/oversulphated chondroitin sulphate remains constant within or between individuals, or if it varies with time, sex, age or atopic status. This would also answer one of the important questions remaining unresolved from this work, that of the possible variation of GAGs in basophils of specific individuals.

Until fairly recently, in vitro systems for the study of human mast cells have not been available; therefore, much of the understanding of normal mast cell function has been derived from rodent models. Within the past several years however, techniques have been developed which permit in vitro investigation of suspensions of human mast cells derived from different anatomical sites, and it has been found that a significant similarity exists between human

and rodent mast cells (Gruber et al 1986; Tharp et al 1987; Schulman et al 1982; Fox et al 1985). The study and characterization of GAG in human mast cells from these different anatomical sites may contribute to the understanding of human mast cell heterogeneity, and their possible function in a given tissue. This would involve the extraction of GAGs from mast cells of the various tissue types, which could then be analysed in terms of their chemical composition and biological characteristics.

The mechanisms involved in the release of histamine from human mast cells and basophils has been the subject of much research in the field of allergy in recent years; the study of GAG release from these cells has however, received very little attention as adequate methods for its measurement were not available. The results described here indicate that GAG may be released from basophils by more than one mechanism. This is an important area which requires further investigation.

It is known from these studies that considerable quantities of GAG are released from basophils in response to allergenic stimulation, but despite exhaustive attempts, the exact percentage of total cell GAG released could not be determined. Methods other than those tested should therefore be sought for the measurement of total cell GAG. The determination of total cell GAG content would also be useful in the study of the exact intracellular location of the GAG contained in basophils and mast cells, and the pattern of its release following activation of the cell. For example, how much cellular GAG, if any, is contained outside the secretory granule, how much is contained in the matrix of the secretory granule, how much is bound to the secretory granule membrane, and exactly how much cellular GAG is released during degranulation of the cells.

Such studies may also indicate whether more than one pool of mediator storage exists in these cells.

Experiments where degranulation is prevented, in the presence of allergen stimulus would be useful to delineate the properties of GAG release further, and to determine whether degranulation of the cell is necessary for its release, or whether GAG is passively secreted without degranulation.

GAGs are metabolized in the liver, and the degradation products excreted by the kidneys. It will therefore also be useful to study GAG derived from mast cells and basophils in the plasma of patients with hepatic and renal dysfunction.

In Vivo Studies Using an Animal Model.

The knowledge gained from the biochemical studies on the Nippostrongylus brasiliensis-infected rat animal model should be used to study further the different cell types in vivo, and their response during systemic anaphylaxis. As rat connective tissue and mucosal mast cells contain different GAGs, analysis of those released will furnish information on

a. Contribution of each type of cell in response to different stimuli.
b. Characteristics of release in relation to protease markers of mast cell type.
c. Mast cell release during antigenic priming.

In addition, when rat basophil GAGs can be characterized, this will permit analysis of basophil involvement in anaphylaxis.

Since the major beneficial function of the basophil/mast cell/IgE system is likely to be the protection of the host against parasitic infections, the study of these cells in patients with helminthic

disease should also be informative and add to existing knowledge of the function of basophils.

Clinical Studies.

It has been assumed for many years, that basophils as well as mast cells are important in the reactions of type 1 hypersensitivity. The study of basophils in such reactions should be continued with clinical studies of both allergy and anaphylaxis. Differences between the basophils and mast cells of patients susceptible to these reactions and those of healthy individuals should be sought and highlighted. The study of basophils in common allergic disease such as asthma and allergic rhinitis is relatively easy to arrange, since this is such a common problem and samples are readily obtained. By contrast, systemic anaphylaxis in humans where basophils would be expected to play a major role is extremely difficult to study, since these reactions are rare, acute and usually unexpected. The most appropriate models for the controlled study of systemic anaphylaxis in humans are likely to be:

a. Hymenoptera venoms (from bee and wasp stings). These reactions may occur during hyposensitization therapy, where patients receive small doses of the venom, which are increased during the course of treatment. Patients are finally challenged with a full dose of the venom, and at this stage anaphylactic reactions may also well occur. Smith et al (1980) previously used this model in the course of a controlled study to evaluate immunotherapy for insect hypersensitivity.

b. Following injection of a drug such as (i) penicillin, for example in patients attending Genito-Urinary Medicine Clinics who frequently receive parenteral antibiotic treatment, or (ii) a

muscle relaxant eg, suxamethonium) or anaesthetic (eg, thiopentone sodium) used preoperatively.

c. Following infusion of blood or blood products, although the resulting reactions are more likely to involve many other factors including complement activation. The mechanisms of these reactions are highly complex and are less likely to reflect a pure IgE-mediated reaction.

Chapter 10.

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Appendix.

List of Publications.

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Robertson DM, Yap PL, Neill G, Reilly KM, Dawes J, Barnetson RStC, Hosking CS. Further studies on the mechanism of adverse reactions to intravenous immunoglobulin. Submitted to Vox Sanguinis.

International Archives of Allergy and Applied Immunology
Editors-in-Chief: L.Å. Hanson, Göteborg; P. Kallós, Helsingborg; F. Milgrom, Buffalo, N.Y.; R.A. Reisfeld, la Jolla, Calif.; K. Rother, Heidelberg; Z. Trnka, Basel; G.B. West, Epsom

Reprint
Publisher: S. Karger AG, Basel
Printed in Switzerland

Int. Archs Allergy appl. Immun. *76:* 286–288 (1985)

0020–5915/85/0763–0286 $ 2.75/0

The Presence in Blood of Both Glycosaminoglycan and Mucosal Mast Cell Protease following Systemic Anaphylaxis in the Rat

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Abstract. The appearance in blood of rat mast cell protease II (RMCPII) and glycosaminoglycan (GAG) was examined in normal and *Nippostrongylus brasiliensis*-primed rats challenged intravenously with worm antigen. Systemic release of these two products occurred only in immune recipients of antigen; substantial levels of RMCPII were also present in the intestinal perfusates of these same rats and there was depletion of both RMCPII and mucosal mast cells (MMC) from the intestinal mucosa. Depletion of MMC was evident after staining for proteoglycan or for serine esterase and the mast cell counts with both histochemical techniques were highly correlated. Taken together, the results suggest that MMC are likely to be the principal source of secreted GAG and RMCPII.

During the immune expulsion of the gastrointestinal nematode *Nippostrongylus brasiliensis* from the rat, there is discharge of granule contents of mucosal mast cells (MMC), and depletion of proteoglycan from these cells [1]. Following intravenous challenge of primed rats with *N. brasiliensis* worm antigen, extensive mucosal damage ensues which is accompanied by the systemic and enteric release of a specific neutral proteinase: rat mast cell protease II (RMCPII) [2] derived from MMC, and depletion of MMC from the jejunal mucosa [3]. The present study was undertaken to analyse body fluids for the presence of MMC-derived proteoglycan during systemic anaphylaxis in the rat.

15 female outbred Wistar rats, immunised 3 and 8 weeks earlier by subcutaneous injection with 6,000 *N. brasiliensis* larvae [4], were allocated randomly to 3 groups of 4 rats and 1 group of 3 rats and were challenged intravenously with 1,000, 500 or 250 worm equivalents (w.e.) of adult whole worm antigen or saline. Worm antigen was prepared as described previously [5]. A further group of 4 naive rats was challenged with 500 w.e. intravenously. 1 h after intravenous challenge, animals were anaesthetised with ether and bled out. Blood was collected into the anticoagulant 'Thrombotect' (Abbott) and the plasma was separated by centrifugation and stored at −70 °C. Serum, small intestinal perfusates and samples of jejunum were collected and the concentrations of RMCPII in these samples were measured by radial immunodiffusion [2, 3]. Additionally, adjacent segments of jejunum were placed in either Carnoy's fluid or 4% paraformaldehyde prepared with phosphate-buffered saline. Tissues were embedded in wax and sections were stained with either Toluidine blue [6] or with naphthol AS-D chloroacetate to demonstrate serine esterases [7]. Plasma, gut perfusates and gut homogenates were analysed for the presence of glycosaminoglycan (GAG) by a modification of the competitive heparin binding assay previously described [8], in which polybrene-Sepharose was substituted for protamine-Sepharose as the binding reagent and the radioactive tracer and standard were heparan sulphate rather than heparin.

RMCPII was present only in the sera of immune rats challenged with worm antigen (fig. 1) and in these same rats there was a 10-fold increase in the concentration of GAG in plasma when compared with immune recipients of saline or naive rats given antigen (fig. 1). Although the release of RMCPII and GAG into the blood was, in this experiment, independent of the dose of antigen, there was a highly significant correlation between the concentrations of RMCPII and GAG ($y = 2{,}110 + 17x$, $r = 0.93$, $p < 0.001$; fig. 2),

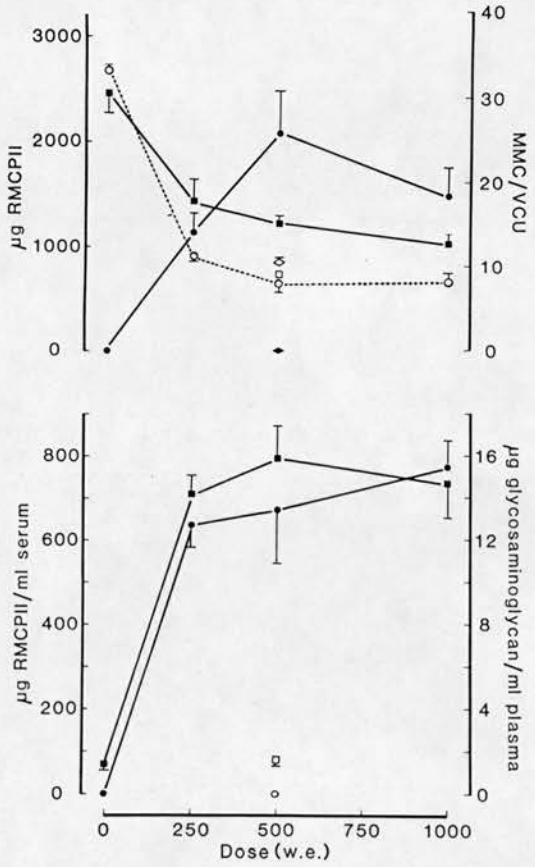

Fig. 1. a Total recoveries of RMCPII (mean ± SEM) in intestinal perfusates (●) and concentration of enzyme in jejunal mucosa (μg/g wet weight tissue, ■) of immune rats given worm antigen or saline (dose = 0). The number of mucosal mast cells (MMC) per villus crypt unit (VCU) detected with Toluidine blue (mean ± SEM, ○) are also shown. Individual symbols showing the concentrations of RMCPII in perfusates (◆) and gut homogenates (□) and the number of MMC (◇) in naive rats challenged with 500 w.e. of antigen are also plotted. **b** The concentrations of RMCPII in serum (mean ± SEM ●) and glycosaminoglycan (GAG) in plasma (mean ± SEM ■) are plotted against the dose of worm antigen. Values from immune rats injected with saline are plotted at the zero dose and in naive rats given antigen the values are plotted at 500 w.e. for RMCPII (○) and for GAG (□).

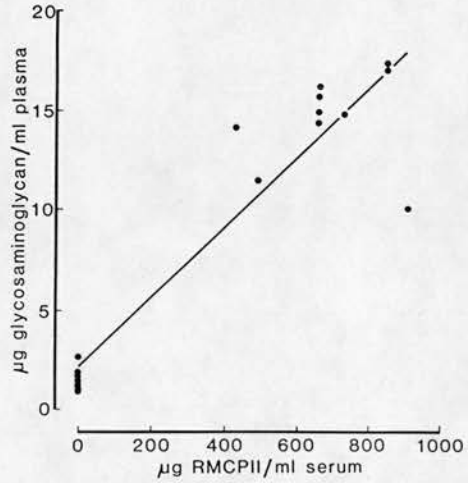

Fig. 2. Regression analysis of the concentrations of RMCPII in serum against GAG in plasma ($y = 2{,}110 + 17\ x$, $r = 0.93$, $p < 0.001$). Plasma was not obtained from one rat given 500 w.e.

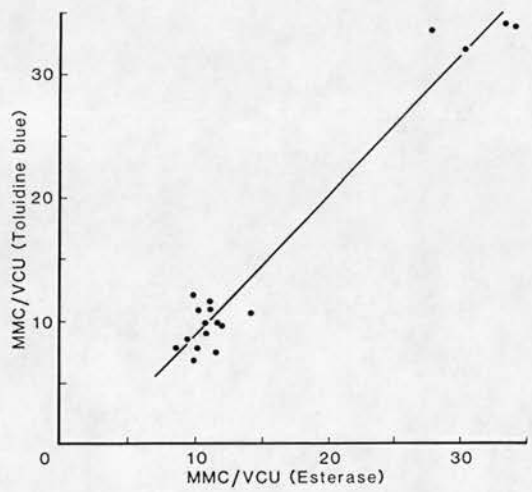

Fig. 3. Regression analysis demonstrating a highly significant correlation between the numbers of MMC enumerated after staining with Toluidine blue and with naphthol AS-D chloroacetate ($y = -2.3 + 1.1\ x$, $r = 0.98$, $p < 0.001$).

which would suggest that they may have been released from the same source.

Intestinal perfusates from primed, challenged rats contained 1,144–2,085 μg RMCPII (fig. 1), whereas perfusates from controls were devoid of this enzyme (fig. 1). There was a concomitant, highly significant ($p < 0.001$) depletion of RMCPII and MMC from the mucosa of primed, challenged rats when compared with control values (fig. 1). Analysis of gut perfusates and homogenates showed GAG to be present within each sample. No decrease from jejunum or increase within the perfusate was detected. The analysis of these tissues is, however, likely to be complicated by endogenous GAG other than that contained in the mast cells.

Mast cell depletion from the mucosa was detected both with Toluidine blue (pH 0.5) and with naphthol AS-D chloroacetate; the correlation between the numbers of mast cells detected with these two methods was highly significant ($y = -2.3 + 1.1\ x$, $r = 0.98$, $p < 0.001$;

fig. 3), suggesting that proteoglycan and serine esterases were depleted concomitantly from discharging MMC.

The results of this study are consistent with data from previous experiments [2] except that shock was severe with the lowest dose of worm antigen and was not augmented by further increasing the challenge dose. There was, as previously described [2], substantial secretion of RMCPII into the gut lumen and a concomitant fall in the mucosal concentration of this enzyme. These changes were paralleled by a reduction in the number of MMC detected with either Toluidine blue or naphthol AS-D chloroacetate. Whilst we were unable to detect changes in mucosal levels of GAG, probably for technical reasons associated with a high background of tissue GAG, there was a tenfold increase in the concentration of GAG in plasma which was highly correlated with the release, into serum, of RMCPII. Although the distribution of RMCPII in tissues other than the gut have not yet been published, our own studies have shown that, apart from the lung in which RMCPII is increased after infection with *N. brasiliensis* and which, at most, contains 300 μg RMCPII/g wet weight, most other organs contain little or none of this enzyme [9]. Clearly, therefore, the major source of secreted RMCPII is the enteric mucosa, and it would seem likely that GAG present in plasma is also released from this site with MMC being the most obvious source of these two products.

More conclusive evidence of the mucosal mast cell as the source of secreted GAG must await further analysis. However, it is relevant to note that MMC granule proteoglycan is, like that from the murine mast cell cultured from bone marrow [10], non-heparin proteoglycan [11], and that the latter cell releases chondroitin sulphate E in response to immunological stimuli [12]. By contrast the heparin present in rat peritoneal mast cells remains within the granule matrix, apparently complexed with the insoluble chymase (RMCPI) [13]. If, as the present study suggests, granule products of MMC are highly soluble, this is yet another example of the biochemical and functional differences between MMC and connective tissue mast cells.

Acknowledgements

S.J.K. thanks the Agricultural and Food Research Council for financial support and *K.R.* is indebted to the Scottish Home and Health Department (grant No. K/MRS/50/660). Funding was also provided by the Medical Research Council (grant No. PG8218470).

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Received: September 7, 1984

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